Alterations in pregnancy-associated glycoprotein concentrations of pregnant sheep experimentally infected with bovine viral diarrhea virus

Andrea Lear DVM, PhD
Ky Pohler PhD
Mee-Ja M. Sula DVM
Matti Kiupel DVM, PhD
Pierre-Yves Mulon DVM
Marc Caldwell DVM, PhD

Received February 14, 2020.
Accepted April 22, 2020.

From the Departments of Large Animal Clinical Sciences (Lear, Mulon, Caldwell) and Biomedical and Diagnostic Sciences (Sula), College of Veterinary Medicine, University of Tennessee, Knoxville, TN 37996; Department of Animal Science, College of Agriculture and Life Sciences, Texas A&M University, College Station, TX 77843 (Pohler); and Department of Pathobiology and Diagnostic Investigation, College of Veterinary Medicine, Michigan State University, East Lansing, MI 48824 (Kiupel).

Address correspondence to Dr. Lear (alear@utk.edu).

OBJECTIVE
To compare pregnancy-associated glycoprotein 1 (PAG1) concentrations in maternal (jugular vein) and fetal (uterine vein) circulations and amniotic fluid samples between pregnant ewes that were and were not experimentally infected with bovine viral diarrhea virus (BVDV).

ANIMALS
11 healthy pregnant yearling ewes.

PROCEDURES
Before study initiation, all ewes were naïve to BVDV and confirmed pregnant by transabdominal ultrasonography at approximately 60 days of gestation. At 65 days of gestation, ewes were intranasally inoculated with a noncytopathic BVDV type 1b strain (concentration, 10^7 $TCID_{50}$/mL; 2 mL/nostril; n = 6) or an equal volume of BVDV-free viral culture medium (control; 5). A blood sample was collected for measurement of PAG1 concentration before inoculation. At 80 days of gestation, each ewe was anesthetized and underwent an ovariohysterectomy. While sheep were anesthetized, blood samples from the jugular and uterine veins and an amniotic fluid sample were collected for measurement of PAG1 concentration. Fetal tissues underwent real-time PCR analysis for BVDV RNA, and placental specimens underwent histologic evaluation and immunohistochemical staining for BVDV antigen.

RESULTS
At 80 days of gestation, BVDV RNA in fetal tissues and mild placentitis were detected in 5 of 6 BVDV-inoculated ewes. Mean PAG1 concentrations in the maternal and fetal circulations of BVDV-inoculated ewes were significantly less than those in control ewes. Mean amniotic fluid PAG1 concentration did not differ significantly between the 2 groups.

CONCLUSIONS AND CLINICAL RELEVANCE
Concentration of PAG1 in the maternal circulation may be a useful biomarker for determining placental health in sheep after viral infection of the reproductive tract. (*Am J Vet Res* 2021;82:63–70)

Bovine viral diarrhea virus is an economically important pathogen of livestock worldwide, and many ungulate species are susceptible to BVDV infection.[1] In pregnant ungulates, the virus can cross the placenta and infect the fetus, and the outcome for the infected fetus is dependent on the immune status of the dam, stage of gestation at the time of infection, and biotype and virulence of the virus.[2] Results of a 2015 meta-analysis[3] indicate that exposure of BVDV-naïve cattle to the virus during gestation results in fetal infection > 95% of the time. Potential reproductive sequelae associated with BVDV infection in pregnant cattle include reduced conception rates, embryonic death, abortion, congenital malformations, and transient or persistent infection of the fetus.[4] Pregnant ewes exposed to BVDV have similar disease progression and outcomes as pregnant cows. However, the age to reproductive maturity is less and the gestation period for sheep is much shorter than those for cattle. Therefore, ewes can serve as a useful experimental model for investigating the pathogenesis of BVDV infection at the maternal-fetal interface.[5]

Pregnancy-associated glycoproteins are produced by the trophoblast and accumulate at the microvillar junction of the placenta.[6,7] Measurement of PAG concentrations in blood and milk is commonly used to determine pregnancy status of ruminants.[8,9] Although the function of PAGs is not completely understood, they have been evaluated as biomarkers for placental health.[6] Decreases in circulating PAG concentrations are associated with embryonic loss and *Neospora caninum*–induced abortion.[10–12] Alterations in PAG concentrations are associated with abnormal placental development in embryo recipients carrying somatic cell nuclear transfer fetuses.[13,14]

ABBREVIATIONS

BVDV	Bovine viral diarrhea virus
Ct	Cycle threshold
CV	Coefficient of variation
PAG	Pregnancy-associated glycoprotein
RT-PCR	Real-time PCR

To our knowledge, variations in PAG concentrations have not been evaluated in pregnant animals following viral infection of the reproductive tract.

The purpose of the study reported here was to determine whether PAG1 concentrations in maternal (jugular vein) and fetal (uterine vein) circulations and amniotic fluid samples differed between pregnant ewes that were and were not experimentally inoculated with BVDV at 65 days of gestation. Our hypotheses were that PAG1 concentrations in all 3 compartments (maternal and fetal circulations and amniotic fluid) of BVDV-infected ewes would be significantly lower than those in sham-inoculated (control) ewes and that the decrease in PAG1 concentrations could be used as an indicator of placental health.

Materials and Methods

Animals

All study procedures were reviewed and approved by the University of Tennessee Institutional Animal Care and Use Committee (protocol 2479). Fifteen yearling primiparous Katahdin ewes were acquired from local sources for the study. The ewes underwent an estrus synchronization program. For each ewe, an intravaginal insert[a] containing 0.9 g of progesterone was placed within the vagina as directed by the manufacturer and removed 7 days later. Dinoprost tromethamine[b] (5 mg, IM) was administered when the intravaginal insert was removed. The ewes were then housed as a group with a reproductively sound ram for breeding by natural service. Transabdominal ultrasonography was used to determine the pregnancy status of ewes 30 and 60 days after breeding activity was first observed. At the 60-day ultrasonographic evaluation, 11 of the 15 ewes were confirmed to be carrying fetuses with a gestational age of approximately 60 days; the remaining 4 ewes were carrying younger fetuses and were excluded from the study.

Experimental design

The 11 pregnant ewes retained in the study were randomly assigned by use of a computerized spreadsheet program[c] to a BVDV-inoculated (n = 6) or control (5) group. Animals were housed indoors within their designated treatment groups. A larger number of ewes were allotted to the BVDV-inoculated group to account for expected fetal loss following virus inoculation. At 65 days of gestation, each ewe of the BVDV-inoculated group was intranasally inoculated with a noncytopathic type 1b BVDV isolate to mimic natural exposure to the virus, and each ewe of the control group was intranasally inoculated with an equal volume of viral culture medium. Blood was collected from each ewe at predetermined times for measurement of serum PAG1 and progesterone concentrations. All ewes underwent an ovariohysterectomy at 80 days of gestation.

Intranasal inoculation

A noncytopathic BVDV type 1b isolate (NY-1 strain) was propagated in Madin-Darby bovine kidney cells in viral culture medium that contained Eagle minimum essential medium with 10% fetal bovine serum, L-glutamine, penicillin G, and streptomycin. The resulting inoculum had a BVDV concentration of 10^7 $TCID_{50}$/mL.

At 65 days of gestation, each ewe was inoculated with the designated inoculum (BVDV or viral culture medium only), as described.[15] Briefly, each ewe was physically restrained and haltered. The ewe's head was then elevated and stabilized for intranasal inoculation. A disposable intranasal cannula was used to deposit 2 mL of the designated inoculum in each nostril. Thus, each ewe in the BVDV-inoculated group received 4×10^7 $TCID_{50}$ of the virus.

Blood sample collection and processing

For each ewe in both treatment groups, a blood sample (approx 10 mL) was collected by jugular venipuncture into a blood-collection tube without any additives at 65 and 80 days of gestation for measurement of serum PAG1 and progesterone (day 80 samples only) concentrations. All blood samples were allowed to clot for 30 minutes and then centrifuged at 3,000 × *g* for 10 minutes. Serum was harvested from each sample, divided into three 1.5-mL aliquots, and stored frozen at –80°C until analysis.

Ovariohysterectomy

At 80 days of gestation (15 days after experimental inoculation), each ewe was anesthetized, positioned in dorsal recumbency, and aseptically prepared for an ovariohysterectomy via a ventral midline incision in a routine manner. A 12- to 15-cm-long incision was made on the ventral midline beginning immediately cranial to the udder attachment and extending cranially. The linea alba was identified and incised. The uterine horns were carefully exteriorized. The uterine vein ipsilateral to the gravid horn was identified, and 3 mL of blood was collected from the vein by use of a 21-gauge butterfly catheter. Then, 3 mL of amniotic fluid was aspirated from the gravid uterine horn by use of a 20-gauge needle attached to a 3-mL syringe. The ovaries, uterus, and all fetal tissues were removed en bloc. A window was created in each broad ligament, and each ovarian pedicle was double ligated 1 cm apart with size-1 or -2 polydioxanone or polyglactin (absorbable) suture prior to transection. The uterine body was ligated at the cervix by use of 1 encircling ligature and 1 transfixing ligature with size-1 or -2 absorbable suture. The body wall was closed in 3 layers as described.[16] The peritoneum and linea alba were closed together in a simple continuous pattern with size-1 absorbable suture, as was the subcutaneous tissue layer. The skin was closed in a Ford interlocking pattern with size-1 polyamide suture. Ewes were allowed to recover from anesthesia following completion of the surgery and were sold at

market after an appropriate drug withdrawal interval had been observed.

All uterine and fetal tissues were placed on ice immediately and processed within 30 minutes after removal from the ewe. Representative placentomes and fetal tissues were flash frozen in liquid nitrogen. All specimens were stored frozen at –80°C until analysis.

PAG analysis

Pregnancy-specific protein B is a placental protein that consists of an unknown number of PAG variants.[17] For the purpose of this study, we refer to pregnancy-specific protein B as PAG1. The concentrations of PAG1 were determined in serum from blood samples collected from the jugular and uterine veins and amniotic fluid samples by use of a commercially available PAG1-specific ELISA,[d] which was performed in accordance with the manufacturer's instructions. The PAG1 concentrations were measured in duplicate for all samples obtained from study subjects as well as a protein standard and serum samples obtained from nonpregnant (negative control) and late-gestation (positive control) ewes. A plate reader set at a wavelength of 650 nm was used to read the plates within 30 minutes after completion of the ELISA, and PAG1 concentrations were derived from a standard curve, which was replicated in duplicate on each plate. The standard best-fit line was accepted with a fit $R^2 > 0.989$. The mean intra-assay CVs for 2 quality control pools were < 3% and < 5%. The mean interassay CVs for those same quality control pools were 2% and 15%.

Progesterone measurement

For serum samples collected from the jugular vein at 80 days of gestation, the progesterone concentration was measured by use of a commercially available radioimmunoassay.[e] The assay was performed in accordance with the manufacturer's instructions, except that an additional 15-minute incubation period was observed before the precipitated solutions were centrifuged. The intra-assay and interassay CVs were both < 10%.

BVDV RT-PCR assay

An RT-PCR assay was used to assess fetal tissue for the presence of BVDV RNA as described.[18] Briefly, fetal tissues obtained from each ewe were homogenized, and RNA was extracted by use of a viral RNA isolation kit[f] in accordance with the manufacturer's instructions. The PRC reaction mixture contained reverse (5'-GYGTCGAACCAYTGACGACT-3') and forward (5'-CCATRCCCDTAGTAGGACTAGC-3') primers and a BVDV probe[g] (5'-TGGATGGCYRAABCCCTGAGT-3' with the addition of dye label 6-FAM on the 5' end and black hole quencher[h] on the 3' end). Any sample with a Ct < 30 was considered positive for BVDV RNA.

Tissue processing and immunohistochemical staining

Placentome specimens were fixed in neutral-buffered 10% formalin and then routinely processed for histologic evaluation. Briefly, formalin-fixed tissue specimens were embedded in paraffin, cut into 4-mm-thick sections, and stained with H&E stain. Additionally, standard immunohistochemical staining procedures were used to assess tissues for BVDV. Paraffin-embedded tissue specimens were sliced into 5-mm-thick sections and placed on charged microscope slides for immunohistochemical staining by an automated system.[i] Each section was deparaffinized and underwent antigen retrieval with a protease 2 retrieval solution for 8 minutes at 37°C. The section was then incubated with a monoclonal anti-BVDV glycoprotein E antibody[j] (concentration, 1:400) for 1 hour. A multimer-based ultramap anti-mouse detection system[k] with red chromogen was used to detect virus-antibody complexes within the tissue specimen. Positive and negative control specimens were processed in a similar manner. Positive control specimens were bovine ear notch specimens that tested positive for BVDV by a PCR assay. Negative control specimens were bovine ear notch specimens for which the pri-

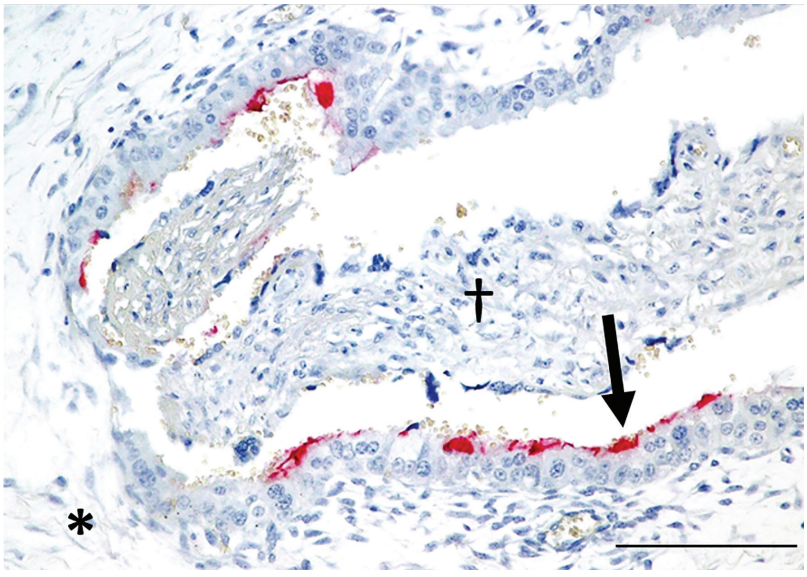
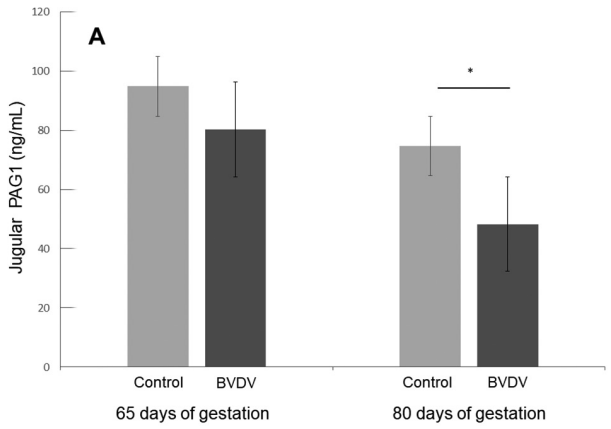
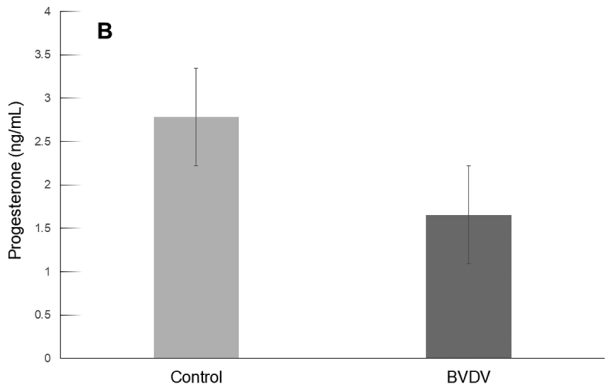


Figure 1—Representative photomicrograph of a section of placentome obtained at 80 days of gestation from a pregnant yearling Katahdin ewe that was experimentally inoculated with 4 X 10^7 $TCID_{50}$ of a noncytopathic BVDV type 1b isolate (NY-1 strain), intranasally, at 65 days of gestation. The chorioallantois (asterisk) is lined by plump trophoblastic epithelium that contains multifocal areas of intracytoplasmic immunoreactivity for BVDV antigen (red-stained tissue; arrow). The maternal endometrium (dagger), which interdigitates with the chorioallantois, is lined by attenuated epithelium that was not immunoreactive for BVDV. Bovine viral diarrhea virus glycoprotein E–specific immunohistochemical stain; bar = 100 µm.





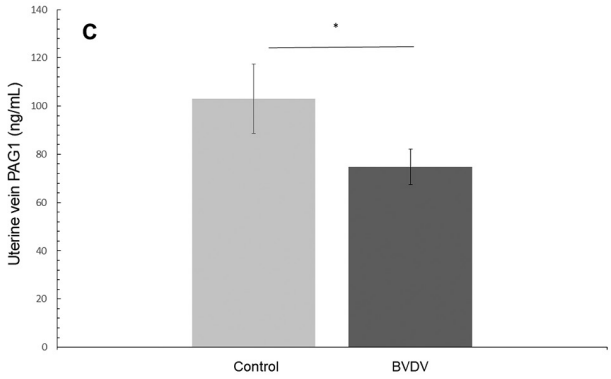


Figure 2—Mean ± SE PAG1 concentration in serum obtained from blood collected from the jugular vein (jugular PAG1 concentration; A) at 65 and 80 days of gestation and mean ± SE progesterone concentration in serum obtained from blood collected from the jugular vein (B) and PAG1 concentration in serum obtained from blood collected from the uterine vein (uterine vein PAG1 concentration; C) at 80 days of gestation for pregnant yearling Katahdin ewes that were experimentally inoculated with 4 X 10^7 $TCID_{50}$ of a noncytopathic BVDV type 1b isolate (NY-1 strain; BVDV-inoculated group; black bars; n = 5) or 4 mL of BVDV-free viral culture medium (control group; gray bars; 5), intranasally, at 65 days of gestation. *Mean differs significantly ($P < 0.05$) between the 2 groups.

mary anti-BVDV antibody was replaced with homologous non-BVDV immune bovine serum.

Statistical analysis

Outcome variables of interest were PAG1 concentrations in serum obtained from blood samples collected from the jugular (jugular PAG1 concentration) and uterine (uterine vein PAG1 concentration) veins and amniotic fluid samples and serum progesterone concentration. The data distribution for each outcome was assessed for normality by means of the Shapiro-Wilk test. All variables were normally distributed, and results were summarized as the mean ± SE. One-way ANOVA was used to compare the serum progesterone concentration at the time of ovariohysterectomy and the uterine vein PAG1 concentration between the 2 treatment groups (BVDV-inoculated and control). A mixed linear model was used to assess the effects of treatment group and sample acquisition time (65 and 80 days of gestation; time) on the jugular PAG1 concentration. The mixed linear model included fixed effects for treatment group, time, and the interaction between treatment group and time and a random effect to account for repeated measures within ewes. The respective associations between serum progesterone concentration and jugular PAG1 concentration at 80 days of gestation and between uterine vein PAG1 concentration and jugular PAG1 concentration were assessed by calculation of the Pearson correlation coefficients. Values of $P < 0.05$ were considered significant. All analyses were performed by use of commercially available statistical software.[1]

Results

Outcome of experimental inoculation

Fetal tissues from all 5 ewes of the control group yielded negative RT-PCR assay results (Ct ≥ 30). Fetal tissue from 1 of the 6 ewes of the BVDV-inoculated group also yielded negative RT-PCR assay results, and data for that ewe were excluded from all subsequent analyses. Fetal tissues from the remaining 5 BVDV-inoculated ewes yielded positive RT-PCR assay results (Ct < 30) with Cts that ranged from 22.47 to 29.29 (mean ± SE, 25.63 ± 2.63).

Histologic evaluation of H&E stained–placentome tissue sections did not reveal substantial differences between BVDV-inoculated and control ewes. Placentome sections obtained from control ewes contained low numbers of mononuclear cells (predominantly lymphocytes) at all levels of the endometrial stroma. In placentome sections obtained from BVDV-inoculated ewes, the intercaruncular regions of the endometrial epithelium contained mildly increased numbers of intraepithelial mononuclear cells, compared with the intercaruncular regions of the endometrial epithelium of control ewes. Evaluation of immunohistochemically stained placentome sections revealed the presence of BVDV antigen within the trophoblastic epithelium lining the chorioallantoic villi of all BVDV-inoculated ewes **(Figure 1)**. Bovine viral diarrhea virus antigen was not detected in immunohistochemically stained placentome sections of control ewes.

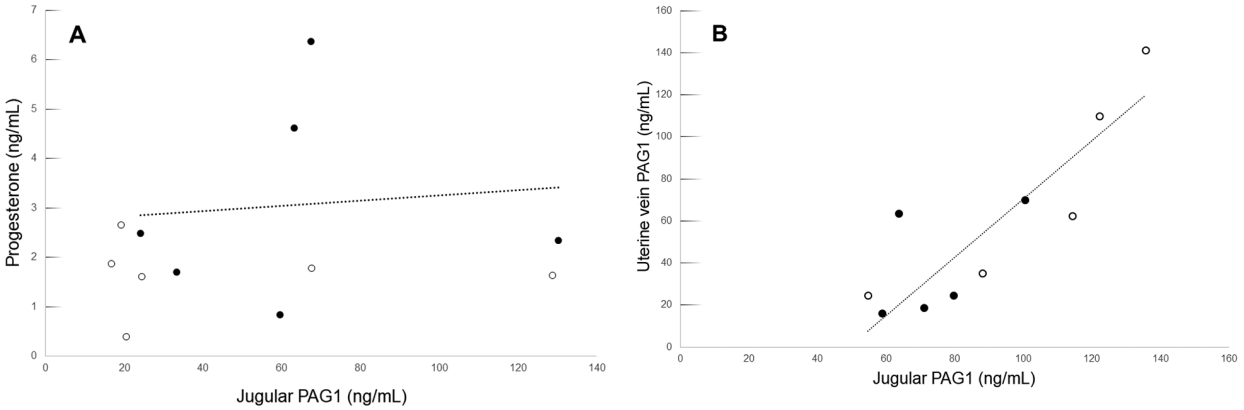


Figure 3—Scatterplots of serum progesterone concentration versus jugular PAG1 concentration (A) and uterine vein PAG1 concentration versus jugular PAG1 concentration (B) at 80 days of gestation for the ewes of the BVDV (black circles) and control (white circles) groups described in Figure 2. Jugular PAG1 concentration was not correlated with serum progesterone concentration ($r = 0.096$; $P = 0.13$) but was positively correlated with uterine vein PAG1 concentration ($r = 0.896$; $P < 0.01$). The dotted line represents the linear regression line. ***See*** Figure 2 for remainder of key.

Progesterone and PAG concentrations

The mean jugular PAG1 concentration did not differ significantly ($P = 0.08$) between the ewes of the BVDV and control groups at 65 days of gestation. However, at 80 days of gestation, the mean jugular PAG1 concentration for the BVDV-inoculated ewes was significantly ($P = 0.04$) less than that for the control ewes **(Figure 2)**. Also, the jugular PAG1 concentration decreased significantly ($P = 0.03$) between 65 and 80 days of gestation, regardless of the treatment group.

The mean serum progesterone concentration at 80 days of gestation did not differ significantly ($P = 0.08$) between BVDV-inoculated and control ewes (Figure 2). The serum progesterone concentration was not significantly ($P = 0.13$) correlated with the jugular PAG1 concentration at 80 days of gestation **(Figure 3)**.

At 80 days of gestation, the mean uterine vein PAG1 concentration for the BVDV-inoculated ewes was significantly ($P = 0.01$) less than the mean uterine vein PAG1 concentration for the control ewes (Figure 2). There was a strong positive correlation ($r = 0.896$; $P < 0.01$) between uterine vein PAG1 concentration and jugular PAG1 concentration (Figure 3). The mean ± SE amniotic fluid PAG1 concentration at 80 days of gestation for the BVDV-inoculated ewes (45.59 ±25.88 ng/mL) did not differ significantly ($P = 0.86$) from that for the control ewes (59.91 ± 2.05 ng/mL).

Discussion

In the present study, intranasal inoculation of pregnant BVDV-naïve ewes with a noncytopathic type 1b strain of BVDV at 65 days of gestation resulted in fetal infection by 80 days of gestation as evidenced by the identification of viral RNA within fetal tissues by RT-PCR assay and histologic evidence of BVDV antigen and inflammation in the placenta. Additionally, for BVDV-inoculated ewes, the PAG1 concentrations in serum obtained from blood samples collected from both maternal (jugular PAG1 concentration) and fetal (uterine vein PAG1 concentration) circulations at 80 days of gestation were significantly lower, compared with those of control ewes that were sham inoculated with viral culture medium at 65 days of gestation. To our knowledge, the present study was the first to describe the effects of BVDV infection on maternal and fetal PAG1 concentrations in a ruminant species.

The intranasal injection method used to experimentally inoculate the ewes of the present study mimicked the most common route of natural exposure to BVDV for pregnant animals.[19] The NY-1 strain of BVDV used to experimentally inoculate the ewes of the present study is a noncytopathic type 1b strain of the virus. Noncytopathic type 2 strains of BVDV have also been used to successfully induce experimental fetal infections in pregnant sheep.[20] Bovine viral diarrhea virus isolates with a noncytopathic biotype are capable of producing an array of reproductive sequelae including the birth of offspring that are persistently infected with the virus.[2] Creation of offspring persistently infected with BVDV requires passage of the virus into fetal compartments and maintenance of the pregnancy by the dam. In the present study, the histologic lesions observed in placental specimens of BVDV-inoculated ewes were minimal, compared with the histologic findings in placental specimens of control ewes; however, immunohistochemical staining confirmed the presence of BVDV antigen in the trophoblasts of BVDV-inoculated ewes. No abortions were detected during the observation period for any of the ewes of the present study. That finding was consistent with the results of other studies[19,20] in which BVDV was used to experimentally induce fetal infections in small ruminant species. In goats, 4 of 9 does experimentally inoculated with a cytopathic strain of BVDV at 65 days of gestation aborted between 7 and 65 days after inoculation.[21] The use of more virulent strains of BVDV for experimental inoculation of pregnant small ruminants may

result in more substantial pathological lesions and dramatic alterations in PAG concentrations in maternal and fetal circulations than those observed in the present study.

The mean serum progesterone concentration for the BVDV-inoculated ewes at 80 days of gestation was numerically but not significantly lower than that for the control ewes in the present study. Infection with BVDV can lead to both oophoritis and fetal death with subsequent release of prostaglandin $F_{2\alpha}$ from the endometrium, and often it is unclear which process has the greatest effect on serum progesterone concentration.[22,23] Within the bovine ovary, there are several cell types that are permissive to BVDV, such as cumulus cells, ovarian stroma, and oocytes throughout their development, but luteal cells appear to be inconsistently affected.[24,25] In nonpregnant BVDV-naïve heifers that were experimentally inoculated with a noncytopathic strain of BVDV, serum progesterone concentrations and luteal tissues appeared to be unaffected by the virus in naturally cycling heifers; however, heifers that had undergone an estrus synchronization program had decreased serum progesterone concentrations during the early diestrus phase of the reproductive cycle and premature luteolysis.[22,23] Embryonic loss, regardless of the cause, is associated with a decrease in serum progesterone concentration owing to indirect luteolysis.[26] Goat does experimentally infected with BVDV at 65 days of gestation developed evidence of oophoritis and luteolysis and had decreasing serum progesterone concentrations between 72 and 135 days of gestation.[21] In that study,[21] 4 of 9 does aborted between 7 and 65 days after BVDV inoculation (ie, between 72 and 130 days of gestation), and it was speculated that luteolysis was induced by either fetal death and the subsequent release of prostaglandin $F_{2\alpha}$ or direct BVDV-induced damage to the luteal tissue. Progesterone is important for the establishment and maintenance of pregnancy. In pregnant ruminants, progesterone is produced by the corpus luteum initially and then by the placenta later in gestation.[27] In sheep, the placenta is the primary source of progesterone production beginning at approximately 50 days of gestation.[28] Histologic examination of the placental specimens obtained from the ewes of the present study indicated that placentitis was minimal at 15 days after BVDV inoculation, which suggested that progesterone production was also likely to be minimally affected in BVDV-inoculated ewes. Ovariohysterectomy at 80 days of gestation precluded investigation of whether the BVDV-induced damage to the placenta would have progressed and eventually impaired progesterone production.

Results of a study[29] involving 31 pregnant ewes indicate that the serum PAG concentration is biphasic, peaking at 184 ng/mL at 63 days of gestation and then decreasing to < 100 ng/mL by 125 days of gestation. A decrease in jugular (maternal serum) PAG1 concentration was observed between 65 and 80 days of gestation for the ewes of the present study; however, the magnitude of that decrease was significantly greater for the BVDV-inoculated ewes than for the control ewes. That finding suggested that the BVDV-inoculated ewes were undergoing trophoblastic injury, which was supported by histologic evidence of inflammation in conjunction with the presence of BVDV antigen within the trophoblastic epithelium and the fact that the mean serum progesterone concentration for the BVDV-inoculated ewes was numerically, albeit not significantly, lower than that for the control ewes at 80 days of gestation. The mean jugular PAG1 concentration did not differ significantly between the BVDV-inoculated and control ewes of the present study at 65 days of gestation prior to experimental inoculation; therefore, the expected decline over time was not the sole reason for the significant difference in jugular PAG1 concentration between the 2 groups at 80 days of gestation. In another study,[6] a pattern of decreasing serum PAG concentrations was described for pregnant ewes fed 2 rations during gestation, with ewes that underwent a noninfectious abortion having a sharper decrease in serum PAG concentration than ewes that carried fetuses to term. In cattle, decreases in serum PAG concentrations have been associated with embryonic loss owing to inadequate ovulatory follicle size, *N caninum* infection, heat stress, high milk production, and fetal death from other unidentified causes.[11,30–32] Because all ewes of the present study underwent ovariohysterectomy at 80 days of gestation, it is unknown whether the jugular PAG1 concentration for the BVDV-inoculated ewes would have continued to decline until abortion occurred or rebounded once the fetus and placenta had cleared the infection.

In the present study, there was a significant strong positive correlation (r = 0.896) between jugular and uterine vein PAG1 concentrations. As expected, the uterine vein PAG1 concentration was greater than the jugular PAG1 concentration owing to dilution or decay of PAGs following entry into the peripheral circulation. The fate of PAGs in the peripheral circulation is unknown. In cattle, the half-lives of PAGs vary from 21.9 hours to 10 days depending on the stage of gestation. It is unclear whether the half-lives of PAGs change in response to alterations in maternal physiology, during periods of fetal stress, or at the time of fetal death. The estimated half-lives of PAGs ranged from 21.9 hours to 3.9 days for pregnant cattle in which embryonic death was experimentally induced at 30 to 38 days of gestation[11,33] and from 8 to 10 days for cattle immediately prior to parturition.[34]

Because PAGs are secretory products of fetal trophoblasts, several functions have been proposed for them. There is experimental evidence that PAGs have immunomodulatory activity that provides some protection for the semiallograft placenta.[6] Results of in vitro studies indicate that PAG1 alters cell proliferation in cultures of bovine bone marrow progenitor cells[35] and induces the release of chemokines in bovine endometrial explants.[36] Pregnancy-associated glycopro-

teins bind uterine serpins (lymphocyte proliferation inhibitors) in vitro, which may further augment their immunomodulatory activity.[37] Accumulation of PAGs in the uterine stroma allows trophoblastic products to directly influence maternal immune cells. Additionally, PAGs have luteotrophic activity. When luteal cells are treated with PAGs, there is an increase in the production of prostaglandin E_2, which is inherently luteotrophic and antiluteolytic in ruminants.[38] Prostaglandin E_2 also inhibits lymphocyte proliferation, which may represent an indirect immunomodulatory mechanism of PAGs.[39] Exposure of luteal cells to PAGs also increases progesterone production, which supports luteal tissue function and pregnancy maintenance.[40] The effects of PAGs at the maternal-fetal interface and elsewhere in the maternal body require further investigation to elucidate their function during pregnancy.

The mean amniotic fluid PAG1 concentration did not differ significantly between the BVDV-inoculated and control ewes of the present study. That finding was unexpected. In another study,[41] PAG concentrations in the fetal compartments of heifers experimentally infected with *N caninum* were significantly greater than those in the fetal compartments of uninfected control heifers. The authors of that study[41] theorized that the increase of PAG concentrations in the fetal compartments of *N caninum*–infected heifers was associated with placental damage and leakage. The BVDV-inoculated ewes of the present study developed minimal placentitis, likely because of the noncytopathic nature and low virulence of the BVDV strain used for experimental inoculation, such that virus-induced injury to the trophoblasts was insufficient to induce a significant increase in amniotic fluid PAG1 concentration.

To our knowledge, the present study was the first to investigate alterations in PAG1 concentrations in maternal and fetal compartments following BVDV infection in a ruminant species. Results indicated that BVDV infection caused trophoblastic injury and decreased the PAG1 concentration in both maternal and fetal (uterine vein) circulations. Those findings suggested that serum PAG1 concentrations can be used as an indicator of placental function during BVDV infection and added to the growing body of evidence that low PAG1 concentrations are associated with placental dysfunction and fetal death. Further research is necessary to investigate the temporal relationship between viral infection of a fetus and alterations in PAG production and function and to elucidate the use of PAGs as biomarkers for assessing the maternal-fetal unit.

Acknowledgments

Supported by a Center of Excellence grant provided by a joint contribution from the state of Tennessee and University of Tennessee.

The authors declare that there were no conflicts of interest.

The authors thank Drs. Aaron Baumann, Samantha Collins, Gessica Franco, and Rebecca Payton for assistance with data collection and laboratory analyses.

Footnotes

a. Eazi-Breed CIDR sheep insert, Zoetis Inc, Parsippany, NJ.
b. Lutalyse injection, Zoetis Inc, Parsippany, NJ.
c. Excel 2016, version 16.0, Microsoft, Redmond, Wash.
d. BioPRYN, BioTracking LLC, Moscow, Idaho.
e. ImmuneChem progesterone kit, MP Biomedicals LLC, Orangeburg, NY.
f. QIAamp Viral RNA Mini Kit, Qiagen, Hilden, Germany.
g. TaqMan, Applied Biosystems, Foster City, Calif.
h. BHQ, Integrated DNA Technologies, Coralville, Iowa.
i. Discovery Ultra automated staining system, Roche Diagnostics, Indianapolis, Ind.
j. BVDV glycoprotein E clone 15.C.5, Syracuse Bioanalytical, East Syracuse, NY.
k. Roche, Ventana Medical Systems Inc, Tucson, Ariz.
l. SAS, version 9.4, SAS Institute Inc, Cary, NC.

References

1. Passler T, Ditchkoff SS, Walz PH. Bovine viral diarrhea virus (BVDV) in white-tailed deer (*Odocoileus virginianus*). *Front Microbiol* 2016;7:945.
2. Kelling CL, Topliff CL. Bovine maternal, fetal and neonatal responses to bovine viral diarrhea virus infections. *Biologicals* 2013;41:20–25.
3. Newcomer BW, Walz PH, Givens MD, et al. Efficacy of bovine viral diarrhea virus vaccination to prevent reproductive disease: a meta-analysis. *Theriogenology* 2015;83:360–365.
4. McGowan MR, Kirkland PD, Richards SG, et al. Increased reproductive losses in cattle infected with bovine pestivirus around the time of insemination. *Vet Rec* 1993;133:39–43.
5. Cleys ER, Halleran JL, McWhorter E, et al. Identification of microRNAs in exosomes isolated from serum and umbilical cord blood, as well as placentomes of gestational day 90 pregnant sheep. *Mol Reprod Dev* 2014;81:983–993.
6. Wallace RM, Pohler KG, Smith MF, et al. Placental PAGs: gene origins, expression patterns, and use as markers of pregnancy. *Reproduction* 2015;149:R115–R126.
7. Wooding FB, Roberts RM, Green JA. Light and electron microscope immunocytochemical studies of the distribution of pregnancy associated glycoproteins (PAGs) throughout pregnancy in the cow: possible functional implications. *Placenta* 2005;26:807–827.
8. Commun L, Velek K, Barbry JB, et al. Detection of pregnancy-associated glycoproteins in milk and blood as a test for early pregnancy in dairy cows. *J Vet Diagn Invest* 2016;28:207–213.
9. Reese ST, Pereira MHC, Edwards JL, et al. Pregnancy diagnosis in cattle using pregnancy associated glycoprotein concentration in circulation at day 24 of gestation. *Theriogenology* 2018;106:178–185.
10. Wallace JM, Aitken RP, Cheyne MA, et al. Pregnancy-specific protein B and progesterone concentrations in relation to nutritional regimen, placental mass and pregnancy outcome in growing adolescent ewes carrying singleton fetuses. *J Reprod Fertil* 1997;109:53–58.
11. Pohler KG, Geary TW, Johnson CL, et al. Circulating bovine pregnancy associated glycoproteins are associated with late embryonic/fetal survival but not ovulatory follicle size in suckled beef cows. *J Anim Sci* 2013;91:4158–4167.
12. Gatea AO, Smith MF, Pohler KG, et al. The ability to predict pregnancy loss in cattle with ELISAs that detect pregnancy associated glycoproteins is antibody dependent. *Theriogenology* 2018;108:269–276.
13. Lee TH, Wu MY, Chen MJ, et al. Nitric oxide is associated with poor embryo quality and pregnancy outcome in in vitro fertilization cycles. *Fertil Steril* 2004;82:126–131.
14. Constant F, Camous S, Chavatte-Palmer P, et al. Altered secretion of pregnancy-associated glycoproteins during gestation in bovine somatic clones. *Theriogenology* 2011;76:1006–1021.
15. Walz PH, Givens MD, Cochran A, et al. Effect of dexamethasone administration on bulls with a localized testicular infection with bovine viral diarrhea virus. *Can J Vet Res* 2008;72:56–62.

16. Noordsy JL. Bovine surgery, in *Proceedings*. 9th Int Cong Cattle Dis 1976;21–30.
17. Roberts JN, May KJ, Veiga-Lopez A. Time-dependent changes in pregnancy-associated glycoproteins and progesterone in commercial crossbred sheep. *Theriogenology* 2017;89:271–279.
18. La Rocca SA, Sandvik T. A short target real-time RT-PCR assay for detection of pestiviruses infecting cattle. *J Virol Methods* 2009;161:122–127.
19. Swasdipan S, Bielefeldt-Ohmann H, Phillips N, et al. Rapid transplacental infection with bovine pestivirus following intranasal inoculation of ewes in early pregnancy. *Vet Pathol* 2001;38:275–280.
20. Scherer CF, Flores EF, Weiblen R, et al. Experimental infection of pregnant ewes with bovine viral diarrhea virus type-2 (BVDV-2): effects on the pregnancy and fetus. *Vet Microbiol* 2001;79:285–299.
21. Desouky HM, Younis AA, Ahmed WM, et al. Experimental infection of pregnant does with bovine virus diarrhoea virus (BVDV): pathological effects on newly born kids. *Glob Vet* 2011;7:415–422.
22. Fray MD, Mann GE, Clarke MC, et al. Bovine viral diarrhea virus: its effects on estradiol, progesterone and prostaglandin secretion in the cow. *Theriogenology* 1999;51:1533–1546.
23. Fray MD, Mann GE, Clarke MC, et al. Bovine viral diarrhoea virus: its effects on ovarian function in the cow. *Vet Microbiol* 2000;77:185–194.
24. Brownlie J, Booth PJ, Stevens DA, et al. Expression of noncytopathogenic bovine viral diarrhoea virus (BVDV) in oocytes and follicles of persistently infected cattle. *Vet Rec* 1997;141:335–337.
25. Fray MD, Prentice H, Clarke MC, et al. Immunohistochemical evidence for the localization of bovine viral diarrhea virus, a single-stranded RNA virus, in ovarian oocytes in the cow. *Vet Pathol* 1998;35:253–259.
26. Gábor G, Kastelic JP, Abonyi-Tóth Z, et al. Pregnancy loss in dairy cattle: relationship of ultrasound, blood pregnancy-specific protein B, progesterone and production variables. *Reprod Domest Anim* 2016;51:467–473.
27. Spencer TE, Bazer FW. Biology of progesterone action during pregnancy recognition and maintenance of pregnancy. *Front Biosci* 2002;7:1879–1898.
28. Senger PL. *Pathways to pregnancy and parturition*. 2nd ed. Redmond, Ore: Current Conceptions Inc, 2005;315–320.
29. Egen TE, Ealy AD, Landon LA, et al. Autoimmunization of ewes against pregnancy-associated glycoproteins does not interfere with the establishment and maintenance of pregnancy. *Animal* 2009;3:850–857.
30. Perry GA, Smith MF, Lucy MC, et al. Relationship between follicle size at insemination and pregnancy success. *Proc Natl Acad Sci U S A* 2005;102:5268–5273.
31. López-Gatius F, Garbayo JM, Santolaria P, et al. Plasma pregnancy-associated glycoprotein-1 (PAG-1) concentrations during gestation in *Neospora*-infected dairy cows. *Theriogenology* 2007;67:502–508.
32. García-Ispierto I, Almería S, Serrano B, et al. Plasma concentrations of pregnancy-associated glycoproteins measured using anti-bovine PAG-2 antibodies on day 120 of gestation predict abortion in dairy cows naturally infected with *Neospora caninum*. *Reprod Domest Anim* 2013;48:613–618.
33. Szenci O, Beckers JF, Sulon J, et al. Effect of induction of late embryonic mortality on plasma profiles of pregnancy associated glycoprotein 1 in heifers. *Vet J* 2003;165:307–313.
34. de Sousa NM, Zongo M, Pitala W, et al. Pregnancy-associated glycoprotein concentrations during pregnancy and the postpartum period in Azawak zebu cattle. *Theriogenology* 2003;59:1131–1142.
35. Hoeben D, Burvenich C, Massart-Leën AM, et al. In vitro effect of ketone bodies, glucocorticosteroids and bovine pregnancy-associated glycoprotein on cultures of bone marrow progenitor cells of cows and calves. *Vet Immunol Immunopathol* 1999;68:229–240.
36. Austin KJ, King CP, Vierk JE, et al. Pregnancy-specific protein B induces release of an alpha chemokine in bovine endometrium. *Endocrinology* 1999;140:542–545.
37. Peltier MR, Liu WJ, Hansen PJ. Regulation of lymphocyte proliferation by uterine serpin: interleukin-2 mRNA production, CD25 expression and responsiveness to interleukin-2. *Proc Soc Exp Biol Med* 2000;223:75–81.
38. Weems YS, Lammoglia MA, Vera-Avila HR, et al. Effect of luteinizing hormone (LH), PGE2, 8-EPI-PGE1, 8-EPI-PGE2, trichosanthin, and pregnancy specific protein B (PSPB) on secretion of progesterone in vitro by corpora lutea (CL) from nonpregnant and pregnant cows. *Prostaglandins Other Lipid Mediat* 1998;55:27–42.
39. Low BG, Hansen PJ. Actions of steroids and prostaglandins secreted by the placenta and uterus of the cow and ewe on lymphocyte proliferation in vitro. *Am J Reprod Immunol Microbiol* 1988;18:71–75.
40. Weems YS, Lammoglia MA, Vera-Avila HR, et al. Effects of luteinizing hormone (LH), PGE2, 8-Epi-PGE1, 8-Epi-PGF2 alpha, trichosanthin and pregnancy specific protein B (PSPB) on secretion of prostaglandin (PG) E (PGE) or F2 alpha (PGF2 alpha) in vitro by corpora lutea (CL) from nonpregnant and pregnant cows. *Prostaglandins Other Lipid Mediat* 1998;55:359–376.
41. Mur-Novales R, Lopez-Gatius F, Serrano-Perez B, et al. Experimental *Neospora caninum* infection in pregnant dairy heifers raises concentrations of pregnancy-associated glycoproteins 1 and 2 in foetal fluids. *Reprod Domest Anim* 2016;51:282–286.