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Using pregnancy associated glycoproteins (PAG) for pregnancy detection at day 24 of gestation in beef cattle

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ABSTRACT

The objective of this experiment was to determine if circulating concentrations of pregnancy associated glycoproteins (PAG) on day 24 of gestation can be utilized to diagnose pregnancy and embryo viability in beef cattle. Postpartum beef cows (n = 677) and heifers (n = 127) were exposed to a 7-day CO-Synch + CIDR estrus synchronization protocol followed by fixed-time AI (FTAI) on day 0. Blood samples were collected at day 24 after TAI to assess circulating concentrations of PAG utilizing an in-house ELISA. Pregnancy diagnosis was performed 30 and 100 days after FTAI via transrectal ultrasonography. Mean circulating PAG concentration at day 24 differed ($P < 0.001$) between animals diagnosed pregnant and non-pregnant at day 30 (1.69 ± 0.10 ng/mL vs 0.30 ng/mL ± 0.07 ng/mL; mean ± SEM; respectively). Pregnant heifers had increased PAG concentration at day 24 compared with pregnant cows ($P < 0.01$; 3.29 ± 0.36 ng/mL vs 1.39 ± 0.10 ng/mL, respectively). Based on receiver operating characteristic (ROC) curve analysis, serum concentration of PAG at day 24 ≥ 0.33 ng/mL in cows and ≥0.54 ng/mL in heifers was 95% accurate at determining pregnancy status at day 30. Heifers that experienced late embryonic mortality between day 30 and 100 of gestation had decreased circulating concentrations of PAG on day 24 (2.02 ng/mL ± 0.73) compared with heifers that maintained an embryo until day 100 (3.69 ng/mL ± 0.39; $P = 0.02$). However, there was no difference in day 24 PAG concentration ($P = 0.39$) between cows that maintained or lost a pregnancy (1.31 ng/mL ± 0.25 vs 0.92 ng/ml ± 0.50). In summary, circulating PAG concentration on day 24 of gestation may be a useful marker for early pregnancy detection in beef cattle, and might be a potential marker for predicting embryonic loss.

1. Introduction

Reproductive failures associated with compromised pregnancy establishment and maintenance are estimated to cost 600 million dollars annually for the beef industry [1]. Pregnancy detection is critical for reproductive management and efficiency of beef herds; however, only 20% of beef producers in the United States do so, while a majority are unaware of the exact pregnancy status of their herd [1]. Early pregnancy diagnosis is key to shortening calving intervals through early identification of non-pregnant animals and timely treatment and rebreeding. Hence, identifying cost effective and accurate methods to determine early pregnancy status in beef cattle may serve as a helpful tool to improve reproductive efficiency of beef herds.

Various methods have been developed to detect pregnancy status in cattle. Transrectal ultrasonography has been used to examine the bovine uterus since the 1980s [2,3] and is currently regarded as the gold standard method for pregnancy detection as it provides visualization of embryo size and heartbeat [4]. However, accurate pregnancy diagnosis using ultrasound is limited to as early as day 27–28 of gestation. Circulating bovine pregnancy associated glycoproteins (PAG) have been shown to be an accurate tool for pregnancy diagnosis in cattle [5–7] and multiple commercial assays are available for detection of PAG in both blood (whole blood, serum and plasma) and milk around day 28 of gestation [8–10]. Pregnancy associated glycoproteins are secreted by binucleated trophoblast cells of the placenta into maternal circulation and are detectable as early as day 24 of gestation [11]. These glycoproteins can be utilized as a biomarker of pregnancy and embryonic viability

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with an accuracy of 95–98% in diagnosing pregnancies between 28 and 30 days of gestation in beef and dairy cows [9–12]. A recent study from our group indicates that dairy cows diagnosed as pregnant on day 31 of gestation have increased circulating concentrations of PAG on day 24 of gestation when compared to those that will be diagnosed as non-pregnant [13]. Therefore, the objective of this experiment was to characterize the differences in serum concentrations of PAG at day 24 post breeding between pregnant and non-pregnant beef females. Additionally, we were interested in determining if circulating concentrations of PAG at day 24 of gestation can be utilized to accurately diagnose pregnancy and to predict embryonic mortality in *Bos taurus* beef cattle. Our hypothesis was that beef heifers and cows diagnosed as pregnant on day 30 of gestation would have increased day 24 circulating PAG concentrations compared to non-pregnant animals, and that females maintaining pregnancies into the second trimester would have increased PAG concentrations on day 24.

2. Materials and methods

All protocols utilized in this experiment were reviewed and approved by the University of Tennessee Institutional Animal Care and Use Committee (IACUC).

2.1. Experiment design

This experiment was conducted at three different locations within the University of Tennessee AgResearch and Education Center system. All animals enrolled in the experiment were maintained on tall fescue pastures with water and mineral ad libitum. A total of 677 postpartum (65 ± 11 ddp) predominantly Angus cows (multiparous $n = 494$; primiparous $n = 183$) and 127 heifers were submitted to a 7-d CO–Synch + CIDR estrus synchronization protocol. Briefly, cows and heifers received a 100 μg injection of GnRH (2 mL Factrel; Zoetis Animal Health, Parsippany-Troy Hills, NJ) and a controlled intravaginal drug release (CIDR) insert (EAZI-BREED CIDR, Zoetis Animal Health) containing 1.38 g of progesterone. Seven days after CIDR insertion, cows and heifers received a 25-mg injection of prostaglandin $F_{2\alpha}$ (5 mL Lutalyse, Zoetis Animal Health) and CIDR inserts were removed. Cows were fixed-time artificially inseminated (FTAI) 66 ± 3 h after CIDR removal with 1 of 8 randomly assigned sires. A second GnRH injection was administered at FTAI. The same procedure was performed with the heifers, however the interval between CIDR removal and FTAI was 54 ± 3 h. The day of FTAI was considered experimental and gestational d 0. Pregnancy was diagnosed based on a viable embryonic heartbeat visualized by transrectal ultrasonography 30 days after FTAI using an Aloka 500V ultrasound (Aloka, Wallingford, CT) with a 7.5 MHz linear probe, and a second examination was performed at day 100 for final pregnancy diagnosis. Cattle considered pregnant by increased PAG concentration at day 24 and diagnosed as non-pregnant at day 30 were considered to have experienced early embryonic mortality (EEM). Cows diagnosed as pregnant at the first pregnancy diagnosis (day 30) and non-pregnant at the second diagnosis (day 100) were considered to have experienced late embryonic morality (LEM).

2.2. Blood sampling and pregnancy associated glycoprotein assay

Blood samples were collected from all cows at day 24 by tail venipuncture using a 10-mL vacutainer tube (BD Vacutainer, Becton, Dickinson and Company, NJ) and allowed to clot at room temperature for 1 h before being placed in a 4 °C refrigerator for approximately 24 h. Samples were centrifuged at 1500×*g* for 15 min and serum stored at −20 °C until later measurement of PAG. Serum concentration of PAG was quantified using an in-house sandwich ELISA established by Green et al. [14] and modified using a polyclonal antibody (Ab 63) as previously described by Reese et al. [13,44]. The sensitivity of the assay for pregnancy detection was 0.33 ng/mL for cows and 0.54 ng/mL for heifers. Each assay was run with duplicates of each sample, a standard curve, a sample from a pregnant cow approximately 60 days into gestation and a sample from non-pregnant cows were utilized as controls. Intra and inter assay coefficients of variation were less than 10%.

2.3. Statistical analysis

The effects of pregnancy status at day 30 and parity on circulating concentrations of PAG at day 24 were analyzed using the MIXED procedure of SAS (SAS 9.4, Institute Inc., Cary, NC). The GLIMMIX (SAS) procedure was used to determine if circulating concentrations of PAG differed on day 24 between cows and heifers that maintained pregnancy compared to the ones that did not maintain between day 30 and day 100. All data were analyzed using the individual animal as the experimental unit, and location as a random variable. Probability for predicting pregnancy by circulating day 24 PAG concentration was determined according to the following equation: probability = $(e^{\text{logistic equation}})/(1 + e^{\text{logistic equation}})$ using LOGISTIC procedure of SAS. MedCalc software was used to create Receiver Operating Characteristic (ROC) curves where day 30 pregnancy status based on ultrasound was designated as the true positive. Predicted cut-off values were chosen based on the optimal criterion, considering not only sensitivity and specificity, but also pregnancy prevalence. Results are presented as mean ± SEM, and differences were considered significant if the $P < 0.05$ while a tendency was described if $0.05 < P < 0.10$. The reported *P*-values were adjusted based on Tukey Kramer's test.

3. Results

Overall pregnancy rate following FTAI at day 30 was 51.8% (416/804) and did not differ (P=0.84) between cows (51.6%) and heifers (52.7%). Heifers and cows diagnosed as pregnant at day 30 had increased ($P < 0.01$) circulating concentrations of PAG at day 24 compared to females diagnosed as non-pregnant (1.69 ± 0.10 and 0.30 ± 0.07 ng/mL, respectively; mean ± SEM; Fig. 1). Day 24 PAG concentration was greater ($P < 0.01$) in pregnant heifers (n = 67; 3.29 ± 0.36 ng/mL) compared to non-pregnant heifers (n = 60; 0.74 ± 0.43 ng/mL). Pregnant cows (n = 349; 1.39 ± 0.10 ng/mL) also had greater ($P < 0.01$) day 24 PAG concentrations when compared to non-pregnant cows (n = 327; 0.22 ± 0.04 ng/mL). There was no significant correlation between day 24 PAG and days postpartum in Pregnant cows. Pregnant heifers had increased day 24 PAG concentration compared to pregnant cows ($P < 0.01$); however, no difference was observed between non-pregnant heifers and cows ($P = 0.10$). Additionally, there were no differences ($P = 0.53$) in PAG concentration at day 24 between primiparous and multiparous cows, hence they were included together in this study. When a logistic regression model was used to predict probability of pregnancy at day 30 of gestation based on day 24 PAG concentration, the probability of pregnancy increased in both heifers and cows as circulating concentration of PAG increased ($P < 0.01$; Fig. 2). The odds of a positive pregnancy diagnosis at day 30 increased 1.5 times in cows and 0.5 times in heifers for every 1 ng/mL increased in PAG concentration at day 24 ($P < 0.01$).

To conduct a more stringent test of the effectiveness of a single circulating PAG concentration measurement to predict pregnancy status, a ROC curve was generated to determine PAG concentrations on day 24 that were accurate in diagnosing pregnancy status at day 30 (Fig. 3). For each parity group, two different curves were

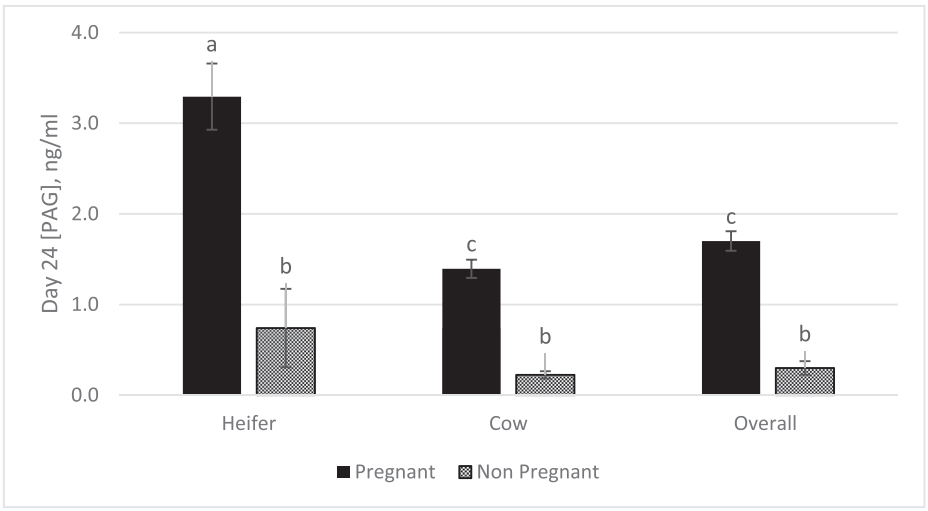

Fig. 1. Day 24 PAG concentration by parity.
Day 24 serum concentration of PAG (mean ± SEM) in heifers (n = 127) and cows (n = 677), that were TAI on day 0 and had a viable embryo on day 30 of gestation. Pregnant heifers had increase ($P < 0.01$) circulating concentration of PAG on day 24 compared with pregnant cows. [abc] Columns with different superscript differ ($P < 0.05$).

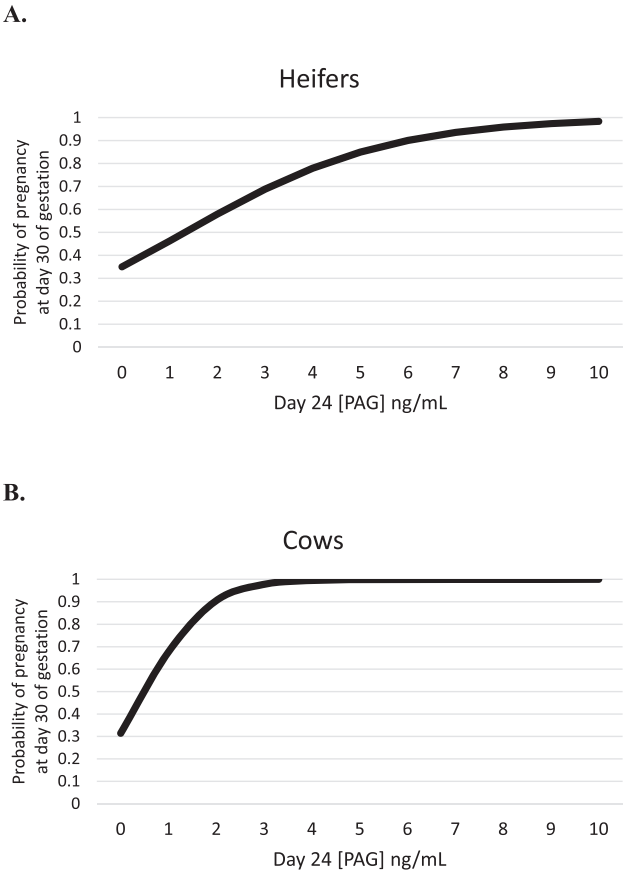

Fig. 2. Probability of Pregnancy at day 30 of gestation.
Probability of pregnancy 30 days after TAI based on day 24 serum concentration of PAG. Increased serum concentration of PAG on day 24 increased ($P < 0.001$) the probability of pregnancy at day 30 of gestation in beef cows and heifers after TAI.

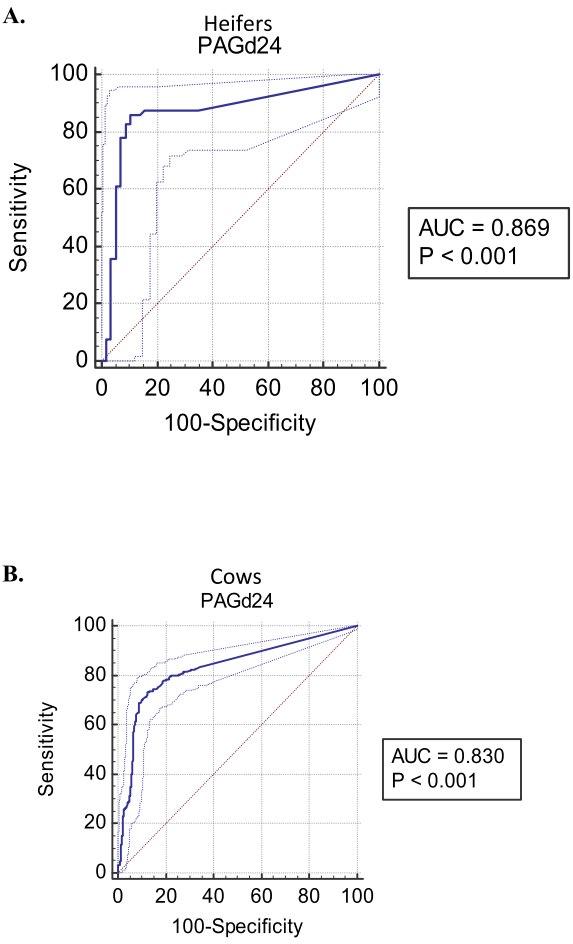

Fig. 3. Day 24 ROC curve to diagnose pregnancy.
Receiver operating characteristic (ROC) curve was used for predictive value analysis. The area under the curve for heifers and cows was 86.9% and 83.0%, respectively, with a positive predictive value at 95% confidence to diagnose pregnancy at day 24 of 0.54 ng/mL for heifers and 0.33 ng/mL for cows.

generated with different cutoff values. The positive predictive cut-off value for heifers was set at 0.54 ng/mL with 90% specificity and 86% sensitivity with an area under the curve of 0.869. For cows, the

predictive cut-off value was set as 0.33 ng/mL with 88% specificity and 73% sensitivity with an area under the curve of 0.830. Based on the ROC curve analysis, 42.8% of cows and 51.2% of heifers were considered pregnant at day 24. However, 91 cows (26.0%) and 9 heifers (13.4%) were considered non-pregnant at day 24 of gestation and diagnosed as pregnant by ultrasound at day 30, therefore, a possible false-positive diagnosis. Heifers and cows that had circulating PAG concentrations greater than 0.54 ng/mL and 0.33 ng/mL (respectively) at day 24 of gestation but diagnosed as non-pregnant by ultrasound at day 30, were considered to have experienced early embryonic mortality (EEM) between day 24 and day 30 of gestation, therefore, a possible false-negative diagnosis. A total of 40 cows (5.9%) and 6 heifers (4.7%) were not pregnant at day 30 after having circulating PAG concentration higher than the cut off values, therefore classified as EEM (Fig. 4).

Late embryonic mortality occurred in 33 animals (8.0%) and was affected by parity ($P < 0.05$) (Fig. 5). Heifers had a greater incidence of LEM compared to cows (14.97 ± 4.71% vs 6.73 ± 1.40%, respectively). Only animals diagnosed as pregnant at day 30 were included in the LEM analysis. Overall, females undergoing late embryonic/early fetal loss between days 30 and day 100 of gestation had decreased PAG concentration at day 24 compared with females that maintained pregnancy up to day 100 of gestation ($P = 0.02$; 1.47 ± 0.47 ng/mL vs. 2.50 ± 0.27 ng/mL; respectively). Heifers that maintained the pregnancy had increased PAG concentration at day 24 (3.69 ± 0.39 ng/mL) when compared to heifers that experienced LEM (2.02 ± 0.73 ng/mL; $P = 0.02$). However, there was no difference in day 24 PAG concentration between pregnant cows that maintained pregnancy and pregnant cows that experienced LEM (1.31 ± 0.25 ng/mL vs 0.92 ± 0.50 ng/mL, respectively; $P = 0.39$).

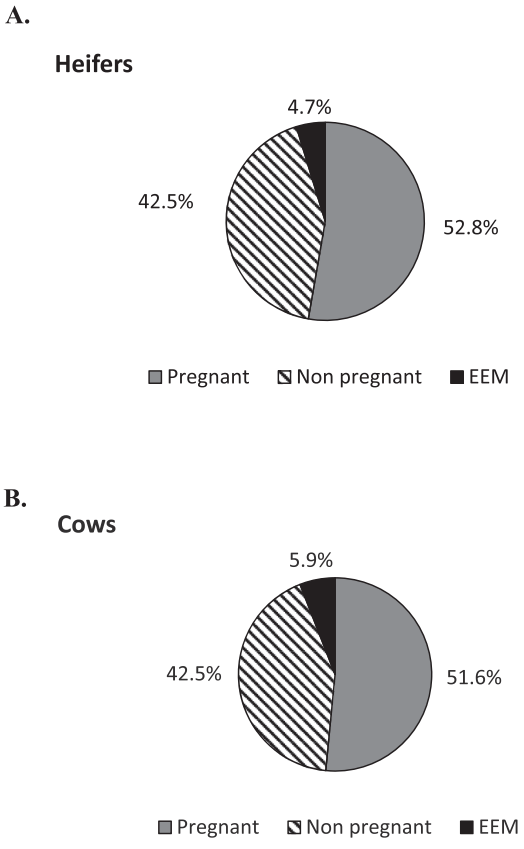

Fig. 4. Pregnancy diagnosis results.
Pregnant animals at day 30 are indicated by the solid grey portion of the pie chart (349 cows and 67 heifers). Cows (n = 40) and heifers (n = 6) that had PAG concentration higher than 0.33 and 0.54 ng/mL, respectively, but were not pregnant at day 30 were considered as EEM (black portion). Non-pregnant cows (n = 288) and heifers (n = 54) are represented by the light shaded portion.

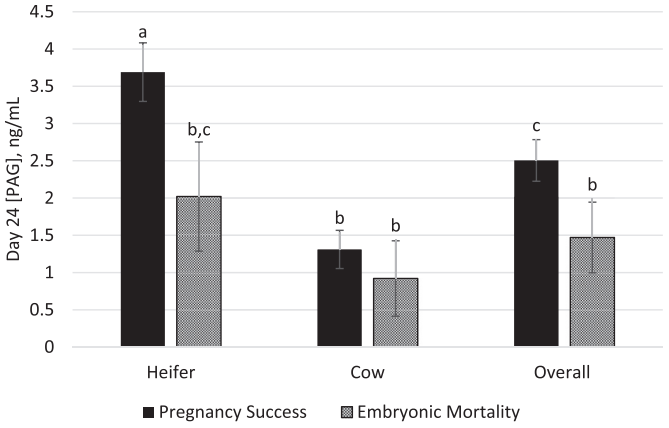

Fig. 5. Day 24 PAG concentration of pregnancy outcome by parity.
Serum concentrations of PAG on day 24 (mean ± SEM) in beef heifers (n = 67) and cows (n = 342) that were diagnosed as pregnant at d 30 and either maintained or experienced embryonic mortality (heifers = 9, cows = 23) by day 100 of gestation. Heifers that experienced late embryonic mortality by day 100 had decreased ($P = 0.02$) circulating concentrations of PAG on day 24 compared with heifers that maintained the pregnancy until day 100. There were no differences between cows that maintained pregnancy and cows that experienced late embryonic mortality ($P = 0.39$). [abc] Columns with different superscript differ ($P < 0.05$).

4. Discussion

Pregnancy diagnosis is a critical part of successful reproductive management practices and early identification of non-pregnancy females would reduce interbreeding and calving intervals by allowing earlier resynchronization and rebreeding. Commercial PAG assay platforms can accurately diagnose pregnancy in cattle and are available in the market for both blood and milk; however, it is limited to day 28 of gestation [9–12]. Research from our group and others have reported that PAG increase in maternal circulation and are detectable around days 24–26 after insemination [11,13,14]. In the present study, circulating PAG concentrations were higher in pregnant compared to non-pregnant beef cows and heifers at day 24 of gestation. Similar results have been reported by Reese et al. [13] where dairy cows undergoing embryo transfer, that were pregnant at day 31, had higher circulating concentration of PAG at day 24 of gestation compared to non-pregnant cows. Additionally, the authors concluded that when circulating day 24 PAG concentrations were greater than 1.39 ng/mL it was 95% accurate in diagnosing pregnancy.

In ruminants, giant binucleate trophoblast cells (BNCs) appear in the uterine luminal epithelium around day 19–21 of gestation [15] and migrate across the microvillar junction to fuse with uterine epithelium. Exocytosis of BNCs delivers their contents (hormones, placental lactogen and pregnancy associated glycoproteins) to the uterine stroma, which enter the maternal circulation across the basement membrane [6,16,17]. However, the exact physiological roles of PAG remain unclear. Several factors have been shown to influence PAG concentration in the maternal circulation, including parity, days post-partum, estrus response, sire and embryonic mortality [18,19]. In the current study, pregnant heifers had increased circulating concentration of PAG compared to pregnant mature cows which has also been reported by others [19–21]. Pregnancy associated glycoproteins have been hypothesized to play

a role in maternal immune modulation and may be luteoprotective based on their ability to induce chemokines, alter neutrophil activity and alter prostaglandin levels in reproductive tissues [22–25]. The increased production of PAG in pregnant heifers might also be influenced by the maternal immune response against the fetus, which is recognized as an allograft during pregnancy [7]. Even though this remains highly speculative, weight and blood volume dilution does not seem to be the contributing factor in this experiment or previously published data [19].

Based on the ROC curve analysis, 42.8% of the cows were considered pregnant at day 24, while 51.2% of the heifers were pregnant at the same day. Additionally, 91 cows and 9 heifers were diagnosed as non-pregnant at day 24 of gestation, however, diagnosed as pregnant at day 30 by ultrasound. Our speculation for the lower pregnancy rates occurring at day 24 compared to day 30 is that some animals may start secreting PAG later than 24 days of gestation. There is significant evidence that individual PAG have distinct temporal secretion patterns with certain PAG genes being expressed earlier in gestation while other being produced during later development. Nevertheless, little information exists on the secretory patterns of different PAG [7,26,27]. This may contribute to the substantial number of animals with a false negative day 24 diagnosis. A total of 5.9% cows and 4.7% heifers had PAG concentrations above the cut off values at day 24 of gestation but were diagnosed as non-pregnant at day 30 in the current study. This does not necessary represents a lack of accuracy in the PAG test. Instead, these animals might have experienced embryonic mortality between day 24 and 30 of gestation, or even prior to day 24 as the antibody used in this assay has previously reported to detect PAG up to 4 days after embryonic death [11]. Similar results have been observed in dairy cattle [13], where 9% of cows had high circulating day 24 PAG concentration, however, were diagnosed as non-pregnant on day 31 of gestation.

In order to increase the accuracy of early pregnancy diagnosis, combining the use of PAG testing with doppler ultrasonography or progesterone testing could potentially improve pregnancy prediction. However, the accuracy associated with diagnosing pregnancy correctly based on the concentration of progesterone varied from 60 to 100% [28,29]. Several reasons for this discrepancy in the accuracy of the milk progesterone test may include an early luteolysis, three verses two follicular waves, a persistent corpus luteum following uterine infection, or a luteal or luteinized cyst. In addition, Pohler et al. [11], reported that serum concentrations of progesterone are not particularly effective at monitoring embryonic viability or the precise timing of embryonic death. Finally, doppler ultrasonography has been incorporated to TAI protocols as a method to detect pregnancy in beef cows as early as day 22 of gestation based on the vascularity of CL and resynchronization of the ovulation on non-pregnant animals [30,31]. Thus, the potential to increase the accuracy by combining certain technologies does exist; however, has not happen to date.

Several reports in the literature suggest that early embryonic loss (before day 30) may vary between 20 and 30% in beef cattle [32,33]; however, it is not clear what the proportion of these losses that are occurring between days 24 and 30 of gestation. Based on the results from this study and previous data [13], it is likely that 5–10% of pregnancy losses in cattle occurs between day 24 and 30 of gestation. These losses could be related to compromised placentation and the requirement for exponential development occurring during this time [34,35]. In the present study, late embryonic mortality (after day 30) was experienced by 6.7% of cows and 14.9% of heifers. Similar results indicate that late embryonic mortality ranges from 4 to 14% in beef cattle [11,36–39] and could be related to number of factors, such as cytoplasmic maturity of the oocyte, source of embryos and placenta insufficiency, all of which may affect the establishment of a functional placenta in cattle [40,41]. Previous studies have shown that increased PAG concentration at around day 30 of gestation are associated with embryonic survival in dairy and beef cattle, suggesting that PAG has the potential to serve as a marker for late embryonic mortality [11,12,19,35,38,39,42]. In this study, females that maintained pregnancy through day 100 of gestation had higher day 24 PAG concentration compared to females that underwent embryonic/fetal mortality. When LEM was compared across parities, only heifers had a significant decrease in circulating PAG concentration at day 24 when experiencing embryonic/fetal mortality. Although late embryonic mortality has decreased incidence compared to early embryonic mortality, it results in serious economic losses to cattle producers because it is often too late to rebreed females that experience late embryonic loss [43].

5. Conclusion

Early pregnancy diagnosis in beef cattle using circulating concentration of PAG at day 24 of gestation has potential and, if applied properly, would reduce the interbreeding and calving interval by allowing earlier resynchronization and rebreeding. Pregnant females had higher day 24 PAG concentration compared to non-pregnant females. Although the detection of PAG is possible earlier in gestation, the benefits as a pregnancy diagnosis tool may be mitigated at this stage by the high incidence of embryonic mortality that occurs after day 24, increasing the number of false positive diagnoses. Circulating concentrations of PAG might be a viable marker not only to determine pregnancy, but also to predict embryonic mortality in cattle. However, further research is needed to improve the predictive value to an acceptable point for use in applied reproductive management.

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