Markers of pregnancy: how early can we detect pregnancies in cattle using pregnancy-associated glycoproteins (PAGs) and microRNAs?

S.T. Reese[1], M.C. Pereira[2], J.L.M. Vasconcelos[2], M.F. Smith[3], J.A. Green[3], T.W. Geary [4], R.F.G. Peres[2], G.A. Perry[5], K.G. Pohler[1,6]

[1]Department of Animal Science, University of Tennessee, Knoxville, TN.
[2]Departamento de Produção Animal, Faculdade de Medicina Veterinária e Zootecnia-UNESP, Botucatu, Brazil.
[3]Division of Animal Science, University of Missouri, Columbia, MO.
[4]USDA-ARS, Fort Keogh Livestock and Range Research Laboratory, Miles City, MT.
[5]Department of Animal Science, South Dakota State University, Brookings, SD.

Abstract

Pregnancy detection has evolved over the last few decades and the importance of early pregnancy detection is critical to minimize the amount of time a cow spends not pregnant. Embryonic mortality (EM) is generally considered to be the primary factor limiting pregnancy rates in cattle and occurs early (<day 28) or late (≥ day 28) during gestation (day 0 = estrus). In cattle, the incidence of early EM is approximately 20 to 40% and the incidence of late EM is approximately 3.2 to 42.7%. Significant effort has been directed toward understanding the mechanisms resulting in early EM up to day 17; however, relatively little is known about the causes or mechanisms associated with EM after day 17. Based on work in these areas, numerous investigators are pursuing methods of early pregnancy or EM detection after day 17 of gestation. This review will highlight some of the technology and markers being used for early pregnancy detection and provide evidence for just how early pregnancy can be detected in the bovine. Advancements in early embryonic or pregnancy detection may lead to development of strategies to overcome early gestation losses.

Keywords: cattle, embryonic mortality, placenta, pregnancy.

Introduction

Successful pregnancy is the most important factor to ensure an efficient and economically sound beef or dairy operation. In order to reach that end point, reproductive loss must be avoided. Early identification of pregnancy failure is key to determining the most effective management strategies and the ability to predict this loss offers greater opportunity to minimize its impact. Loss of pregnancy may occur at any time between conception and calving; however, some time points are more critical than others. Pregnancy failure affects all cattle; however, high producing dairy cattle are more susceptible to decreased pregnancy rates than dairy heifers and beef cows (Diskin *et al.*, 2011; Pohler *et al.*, 2015a; Pohler *et al.*, 2016a, b). Although fertilization rate in cattle is often greater than 85% (Santos *et al.*, 2004, Diskin and Morris, 2008) there have been reports of differences in fertilization rates in beef and dairy cattle that result in a large amount of reproductive failure (Breuel *et al.*, 1993, Sartori *et al.*, 2002; Santos *et al.*, 2004, 2009); however, the focus of this review will be on post fertilization failure and detection. In addition, fetal losses (>day 45 of gestation) are low at approximately 3% (Inskeep and Dailey, 2005). Thus, reproductive loss during the embryo stage (day 0 to 44) of development is substantial. Early embryonic loss can be classified as loss that occurs before day 28 of gestation. Although the embryonic heartbeat can be detected by this time via real-time ultrasonography, the conceptus does not yet resemble a calf. Causes of early embryonic mortality include lethal genetic mutations, uterine asynchrony, and maternal recognition failure (Ayalon, 1978; Diskin and Morris, 2008). Early embryonic loss is generally accepted to account for 20 to 40% of pregnancy failure (Sreenan and Diskin, 1986; Inskeep and Dailey, 2005; Santos *et al.*, 2004, 2009). During the late embryonic period, through day 44, growth can be characterized by the development of limb buds, eye orbits and the formation of placentomes. Although late embryo mortality accounts for less than 10% of pregnancy loss, it has significant implications for the producer and has been suggested to cause greater financial burden then early EM (Diskin and Morris, 2008). By day 45, the conceptus takes the true form of a fetus with split hooves, ribs and displays limited movement (Curran *et al.*, 1986). After days 45 to 60 pregnancy loss decreases and is less than 5% through the second and third trimester of pregnancy.

Pregnancy failure is extremely costly to the producer. Lost revenue can be attributed to cost of feeding and managing nonpregnant cows, decreased weight of late born calves at sale time and a decreased calving percentage due to cows that lost pregnancy. In a study involving lactating dairy cows, pregnancy loss after 1 month of gestation cost producers an average of $555 (US) due to repeat breeding expenses, increased calving interval and increased probability of involuntary culling (De Vries, 2006). In beef cattle that have had pregnancy loss and manage to become pregnant to a subsequent breeding, there is still a significant amount of lost revenue from reduced weight of late born calves and decreased uniformity of the calf crop. Pregnancy diagnosis is a very important management tool that is underutilized in the United States. According to the USDA's 2008 National Animal Health Monitoring System (NAHMS) Beef survey, only 20% of operations

utilize pregnancy diagnosis via palpation or ultrasound, a number that has remained virtually unchanged since the 1997 survey (United States Department of Agriculture - USDA, 2010). In comparison, the 2007 NAHMS Dairy survey reported 93% of operations perform pregnancy detection (USDA, 2009). Pregnancy diagnosis can identify open cows, help estimate calving dates, and help producers make culling decisions. This review will describe various methods of pregnancy diagnosis in cattle and research advancements that may allow for earlier detection of pregnancy.

Established methods of pregnancy diagnosis

Rectal palpation and ultrasonography

As the conceptus develops during gestation, fluid accumulates, and placentation advances, methods of pregnancy detection allow for manual transrectal palpation of the uterus per rectum and its contents. Transrectal palpation of the uterus, starting as early as day 35 of gestation allows for detection of a pregnancy by palpation of fluid and the amniotic vesicle within the uterus. Palpation of the uterus and its contents is traditionally practiced from 40 to 60 days after insemination with the earliest detection limit being approximately 30 to 35 days post insemination. Additional sensitivity can be achieved during this time point by using transrectal ultrasound for pregnancy detection (Lucy *et al.*, 2011). Ultrasound is the gold standard for determining pregnancy and confirming the presence of a viable embryo. Transrectal ultrasonography can be accurate as early as day 26 to 29 to diagnose pregnancy and visualize a discernable heartbeat (Pierson and Ginther, 1984; Kastelic *et al.*, 1988; Beal *et al.*, 1992). Doppler ultrasound may provide additional information based on visualization of blood flow to the placenta/conceptus; however, data supporting its use as a pregnancy diagnosis method has been mixed. Today, ultrasound is considered the only visual indicator of pregnancy in cattle and is used for comparison with all recent attempts at diagnosing earlier pregnancy in this review. With all palpation and ultrasound techniques, a highly experienced individual is required to complete these test.

Chemical based pregnancy tests

Earlier and more effective means of pregnancy diagnosis are constantly being sought and evaluated. Numerous chemical and biochemical based pregnancy tests have been developed and tested for use in cattle. Each has strengths and shortcomings that have led to their adoption or lack of use in various production schemes. One important consideration in evaluating pregnancy diagnosis tests is the difference between pregnancy specific and non-pregnancy specific methods. Pregnancy specific markers are physiologically present only in pregnant animals and produced specifically from the pregnancy; whereas non-pregnancy specific markers, while elevated during pregnancy, may be produced under other physiological conditions as well.

Progesterone is one example of a non-pregnancy specific diagnosis method. Progesterone is one of the more common chemical based pregnancy tests commercially available although overall a very small percentage of producers use it (USDA, 2009). Produced by the corpus luteum (CL), progesterone is a steroid hormone that is crucial for maintaining pregnancy; however, the cyclic profile of progesterone mandates that pregnancy detection must occur between luteolysis and the formation of a new CL. During this time period, non-pregnant cows should exhibit low progesterone levels, whereas progesterone concentrations in pregnant cows should remain elevated. Significant differences in progesterone concentration appear between pregnant and non-pregnant cows between day 20-24 post insemination in both serum and milk. Accurate positive pregnancy diagnosis varied between 60 and 100% for milk progesterone, however detection of non-pregnant animals varied between 81 to 100% (Nebel *et al.*, 1987; Sasser, 1987, Nebel, 1988). Longer luteal phases in some cows, cysts or persistent follicles may play a role in elevated progesterone concentrations during the pregnancy test period that yield false positive results (Pohler *et al.*, 2015a). Some discrepancies exist in evaluating the effectiveness of progesterone testing with regards to embryo loss. Research has shown that pregnant cows with progesterone concentration below 3.76 ng/ml at week 5 were more likely to experience embryonic mortality before week 9 than cows with a greater concentration of progesterone (Starbuck *et al.*, 2004). However, it should be noted that a majority of cows (77%) in the low concentration group maintained pregnancy through week 9. A later study by Pohler *et al.* (2013), demonstrated that serum concentration of progesterone between day 28 to 30 in pregnant cows was not predictive of pregnancy loss between a positive pregnancy diagnosis by progesterone and final pregnancy confirmation at day 70 of gestation.

Another steroid hormone, estrone sulfate is produced by the conceptus and can be detected at day 100 of pregnancy in cattle (Holdsworth *et al.*, 1982). Estrone sulfate is a pregnancy specific marker, though its late period of detection limits its use in domestic cattle. Although steroid hormone pregnancy tests have largely been replaced in cattle, sheep and swine, use of estrone sulfate has been used for pregnancy detection of non-domestic animals in the wild and zoos through noninvasive fecal and urine samples. Estrone sulfate has been successfully evaluated for use in pregnancy detection in hoofed stock, gorillas, orangutans, baboons and wild felids (Kumar *et al.*, 2013).

Early pregnancy factor (EPF), also known as early conception factor, appears in maternal circulation shortly after fertilization. In cattle, EPF is observed within 48 h after breeding and seems to contribute to maternal immune suppression and implantation preparation (Morton, 1998; Cordoba *et al.*, 2001). A study by Athanasas-Platsis, *et al.* (1989) demonstrated a critical role of EPF when mice that had been immunized against EPF had decreased embryo viability and an

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increased incidence of pregnancy failure. Action of EPF is not confined to pregnancy as growth factor-like properties have been seen in tumors. A large percentage of embryo loss occurs after the recommended sampling time of commercial EPF assays (48 h to 7 days). Studies have indicated that commercial EPF tests have a sensitivity (or detection of pregnant animals) of 45 to 86% and a specificity (or detection of non-pregnant animals) of 4 to 28.8% (Cordoba *et al.*, 2001; Gandy *et al.*, 2001). In regards to EPF as a pregnancy detection method, commercial tests are unreliable at identifying non-pregnant animals that limits its use in this capacity.

The maternal recognition of pregnancy signal in cattle and other ruminants is interferon tau (IFNT). Interferon tau stimulates CL maintenance through endocrine-like actions, blocks estrogen receptors and paracrine mechanisms on the endometrium prompt IFNT stimulated gene production (Pohler *et al.*, 2015a, Spencer and Hansen, 2015). Due to the difficulty associated with detecting small concentrations of IFNT directly, research has focused on measuring IFNT-stimulated genes (ISGs) that have been upregulated in peripheral mononuclear blood cells (PBL). Conceptus IFNT mRNA concentration peaks in cattle at day 20 post-conception (Han *et al.*, 2006; Spencer and Hansen, 2015). Genes including ISG15, Mx1 and Mx2 are more highly expressed in peripheral blood leukocytes (PBL) of pregnant cows than non-pregnant cows (Han *et al.*, 2006; Gifford *et al.*, 2007). A study by Green, *et al.* (2010), showed that pregnant heifers had greater IFNT-stimulated gene expression than cows. They concluded that a IFNT-stimulated gene based pregnancy test would be possible for heifers at day 18 but not for cows whose response is more limited based on the current sensitivity of available assays (Green *et al.*, 2010). Unfortunately, ISGs are not unique to pregnancy which limits their use as a pregnancy detection tool to identifying non-pregnant animals (Han *et al.*, 2006; Gifford *et al.*, 2007; Pohler *et al.*, 2015a); however, a resynchronization protocol can be implemented in cows that are identified as non-pregnant which improves operation efficiency (Lucy *et al.*, 2011).

MiRNAs: potential biomarkers for pregnancy diagnosis

The search for easily accessible biomarkers of various diseases and physiological states has recently focused on circulating microRNAs (miRNA). Between 18 and 22 nucleotides in length, miRNAs play important roles in regulation of gene expression and have been found in biological fluids ranging from serum and amniotic fluid to urine and milk (Reid *et al.*, 2011; Pohler *et al.*, 2015a). MicroRNAs are released from cells of most tissue types in plasma membrane bound extracellular vesicles (EV), especially exosomes. The packaging of miRNA in EVs or exosomes is important from a detection standpoint as RNA-ases are unable to penetrate and breakdown the miRNA allowing them to be extracted from blood and serum (Reid *et al.*, 2011). Exosomes and EVs play a crucial role in intercellular communication, including promotion of sperm

maturation, regulation of immune function, release of miRNA for a wide array of regulatory functions, as well as other roles currently being studied (Raposo and Stoorvogel, 2013). Serum and whole blood have proved an acceptable source of EV-derived miRNA profiles, thus providing a potential blood-borne biomarker candidate for various disease and physiological states (Häusler *et al.*, 2010; Reid *et al.*, 2011). Human based disease research has revealed significant differences in miRNA abundance for many cancers (Lawrie *et al.*, 2008; Häusler *et al.*, 2010), heart disease (Tijsen *et al.*, 2010) and sepsis (Wang *et al.*, 2010). In addition, circulating miRNAs in maternal serum have been observed as potential biomarkers of pregnancy status due to their significant impact on gene expression and regulation (Chim *et al.*, 2008). A study by Gilad *et al.* (2008) identified miRNAs that are increased in abundance in pregnant humans but not in non-pregnant females. This finding led to the rapid expansion of identifying miRNAs that were specific to pregnancy and across various species, although none have been thoroughly explained.

An initial study of pregnancy specific markers in mares identified 7 miRNAs that were only expressed in pregnant mares (Cameron *et al.*, 2011) compared with the non-pregnant controls. In addition, work in the sheep has confirmed the presence of miRNA in uterine lumen fluid in pregnant and cyclic sheep (Burns *et al.*, 2014). These data support the idea for a likely role of miRNA in conceptus-endometrial interactions during the establishment of pregnancy (Burns *et al.*, 2014). In addition, a follow up study to the one described above, provides evidence that EVs are produced from the trophectoderm and uterine epithelia in the pregnant ewe and are involved in intercellular communication (Burns *et al.*, 2016).

Many groups are now looking into miRNAs as biomarkers for pregnancy detection in the cow. There is increasing evidence that pregnancy specific miRNAs exist and may be potential markers for pregnancy diagnosis. In 2015, exosomal miRNAs were reported to be differentially expressed in pregnant versus non-pregnant cows and cows undergoing early embryonic mortality (Pohler *et al.*, 2015b). A recent study by Fiandanese *et al.* (2016) identified a potential miRNA, bta-mir 140, as an early biomarker for pregnancy detection. At day 19, bta-mir 140 was up regulated in all pregnant cows, and at day 13 onwards, it was upregulated in pregnant, non-lactating cows (Fiandanese *et al.*, 2016). Similarly, Ioannidis and Donadeu (2016) identified 6 miRNA (day 16: bta-miR-26a, bta-miR-29c, bta-miR-138, bta-miR-204. Day 24: bta-miR-1249, day 16 & 24: hsa-miR-4532) that were differentially expressed in pregnant heifers. Although refinement is necessary to pinpoint ideal miRNA for pregnancy diagnosis, results indicate that miRNAs have potential as an early pregnancy detection tool. Furthermore, miRNA may provide information to denote embryonic viability. Preliminary data from our laboratory indicate cows that experience embryo mortality compared to cows that have a successful pregnancy have a significantly increased abundance of specific miRNAs at days 17 and 24 of

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gestation. Future studies are needed to assess the repeatability of these findings and to determine precise miRNA most applicable for embryo viability analysis.

Developing early pregnancy diagnosis method; pregnancy associated glycoproteins

The placenta

Proper placentation is crucial for pregnancy development and ultimately pregnancy success. Active placentation in the cow occurs between day 28 and 40 of gestation (Aires *et al.*, 2014). Bovine placentation involves adhesion between the maternal-caruncle structures and fetal cotyledonary tissues to form placentomes. Superficial interdigitation begins around day 20 in cattle between microvilli of the trophectoderm and uterine epithelium. True placentomes, marked by increased villi length and raised tissue in caruncular endometrium are distinct by days 31 to 33 of gestation. By day 39, placentomes are easily discernable and have long, occasionally branching villi (King *et al.*, 1979).

Binucleated trophoblast cells (BNCs) appear in the fetal chorion of ruminants at days 18 to 19 of gestation and comprise 15 to 20% of the trophectoderm throughout pregnancy. Binucleated trophoblast cells migrate to the maternal epithelium from the fetal chorion after maturation but do not penetrate past the basement membrane (King and Atkinson, 1987). Contact between the maternal and fetal interface at the microvilli junction allows migration of BNC's towards the basement membrane to begin (Wooding and Wathes, 1980, Wooding and Burton, 2008). Products of BNC's, including hormones, placental lactogen and pregnancy associated glycoproteins (PAGs), are packaged in secretory granules and enter maternal circulation across the basement membrane (Pohler *et al.*, 2015a).

PAG production

Pregnancy associated glycoproteins were identified in the 1980's during early attempts to develop pregnancy-specific markers that could be used for pregnancy diagnosis. Although PAGs are often thought of to be produced by BNCs, Green *et al.* (2000) reported that PAGs can be sorted into two separate families based on their expression in trophoblast cells. Some PAGs are expressed in both BNCs and mononucleated trophoblast cells while others are solely produced in BNCs (Green *et al.*, 2000). Although their physiological role is unknown, a large number of distinct PAGs and more than two dozen specific PAG genes have been described. Based on accumulation of PAGs at the junction between uterus and placenta and known proteolytic activity of certain PAGs, it has been hypothesized that PAGs may help process growth factors or may have adhesion actions (Wallace *et al.*, 2015). Based on evidence that PAGs may inhibit certain immune cells, they may also play a role in disguising antigens from the maternal immune system (Perry *et al.*, 2005). After appearance of BNCs and epithelial adhesion of trophoblast, the first sizable increase in

PAG concentration occurs between days 22 to 24 of gestation. Concentrations of PAG continue to increase through day 36, followed by subsequent decrease in concentration until day 60 of pregnancy followed by a steady increase through the second and third trimesters of pregnancy. In the weeks preceding parturition, a substantial increase in circulating concentrations of PAG occurs that peaks at calving. This may be attributed to significant placental growth at the end of gestation or the release of stored PAG from other tissues (Green *et al.*, 2005; Pohler *et al.*, 2013). Eight weeks post parturition, PAGs are not detectable in maternal circulation (Green *et al.*, 2005).

PAGs and pregnancy diagnosis

Since their discovery, PAGs have been a target for pregnancy diagnosis. Pregnancy-specific protein B was the first identified PAG of interest by scientists looking for pregnancy specific markers that could be detected early in gestation in the 1980's (Butler *et al.*, 1982; Sasser *et al.*, 1986). Using early assays, PAGs were detectable at day 24 of gestation in cattle; however, the physiological function was unknown which is still the case today. Discovery of multiple PAG families and genes has contributed to understanding the increasing complexity of PAGs. The radioimmunoassay (RIA) first developed shortly after discovery of pregnancy specific protein B was the standard for PAG detection for many years (Zoli *et al.*, 1992). This assay was highly specific and the validating study concluded that PAGs were secreted into the maternal system and were unique to pregnant animals. A study by Green *et al.* (2005) validated an ELISA that specifically targeted PAGs secreted early in gestation that had a shorter half-life (4.3 days *vs.* 8.4 days) than the previous targets to reduce the potential for false positives in postpartum cows (Zoli *et al.*, 1992; Sousa *et al.*, 2003; Green *et al.*, 2005). The ELISA was demonstrated to accurately detect pregnant cows via serum concentrations of PAGs at day 28 post insemination. Studies comparing the efficacy of the PAG ELISA, PAG RIA and transrectal ultrasonography revealed comparable results for the diagnosis of pregnancy in cattle at day 28 of gestation although some differences were identified in the ability of certain assays to detect non-pregnant animals (Szenci *et al.*, 1998; Karen *et al.*, 2015).

Commercial PAG tests are currently available using both milk and blood samples, and include BioPRYN (BioTracking LLC. Moscow, ID USA), IDEXX Bovine pregnancy test (IDEXX Laboratories Inc. Westbrook, ME USA) and DG29 pregnancy test (Genex Cooperative Inc. Shawano, WI USA). BioPRYN accepts blood samples from heifers 25 days post breeding and cows 28 days post breeding, IDEXX recommends day 28 blood or milk samples and DG29 has been validated using day 29 blood samples. At the recommended sampling time, all commercial tests provide 98-99% true positive (pregnant) reading and false positive (reported as pregnant but open) rates range from 1-5% however, some variation may be due to late embryonic mortality.

Early pregnancy diagnosis

Current research is focused on finding markers and increasing sensitivity to identify pregnant cows before day 28 of gestation. Although diagnosis is limited to the time frame following the introduction of PAGs to maternal circulation at days 19 to 20, preliminary research indicates that PAGs may be effective at diagnosing pregnancy as early as day 24. Currently, research is focused on heifers, which exhibit greater PAG concentrations earlier in gestation compared with cows. A recent study in Brazil evaluated the accuracy of pregnancy diagnosis at day 24 in predominantly Holstein heifers following timed embryo transfer (Reese *et al.*, 2016). Serum PAG concentrations at day 24 differed between pregnant (2.98 ng/ml) and non-pregnant (0.69 ng/ml) heifers. Using receiver operating curve analysis, PAG concentrations greater than 1.39 ng/ml were 95% accurate in diagnosing a pregnant heifer at day 24 of gestation. Using a day 17 baseline sample, the difference between day 24 and day 17 samples predicted 79% of pregnancies. This, early pregnancy diagnosis using PAG is possible; however, more work is needed in this area. It is realistic to assume that day 24 PAG concentrations can diagnosis pregnancy, but high rates embryonic loss before the standard day 30 tests may decrease the efficiency and benefits of testing early. In a recent study, embryonic mortality between day 24 and 31 of gestation was 20.8% in lactating dairy cows, thus pregnancy loss following maternal recognition of pregnancy may be more prevalent than previously thought making early pregnancy diagnosis less useful (Pohler *et al.*, 2016a).

PAGs as indicators of embryo success

Recent studies have demonstrated a strong correlation between successful pregnancies and PAG concentrations during early gestation. Increased circulating PAG concentrations approximately day 28 of gestation are generally predictive of increased embryo survival, making PAG a likely marker for evaluating embryo viability and placental competence. In comparison to progesterone, which exhibit no difference between heifers or cows that undergo embryo mortality and those that maintain pregnancy, PAG concentrations are significantly different between heifers and cows (Fig. 1; Kill *et al.*, 2013). Serum PAG concentrations in cows that maintained pregnancy (4.53 ± 0.34 ng/ml) were significantly higher than cows that underwent pregnancy loss (3.14 ± 0.72 ng/ml) after fetal heartbeat detection at day 28 (Pohler *et al.*, 2013). All cows had a pregnancy with a fetal heartbeat at day 28, indicating a viable pregnancy at that time. Perhaps more importantly, PAGs seem to be particularly effective at identifying cows that will undergo late embryonic or early fetal mortality. As serum concentrations increased, the probability of embryo mortality significantly decreased. Late embryo mortality between days 31 and 59 was predicted with 95% accuracy if PAG concentrations were <1.4 ng/ml at day 31 after timed artificial insemination (Pohler *et al.*, 2016a). Pohler *et al.* (2016b) demonstrated that both *Bos indicus* and *Bos taurus* cows that experienced embryo mortality had similar and lower PAG concentration at day 28 despite the significant differences between PAG concentrations of successful pregnancy in B*os taurus* compared with B*os indicus* cows (Fig. 2).
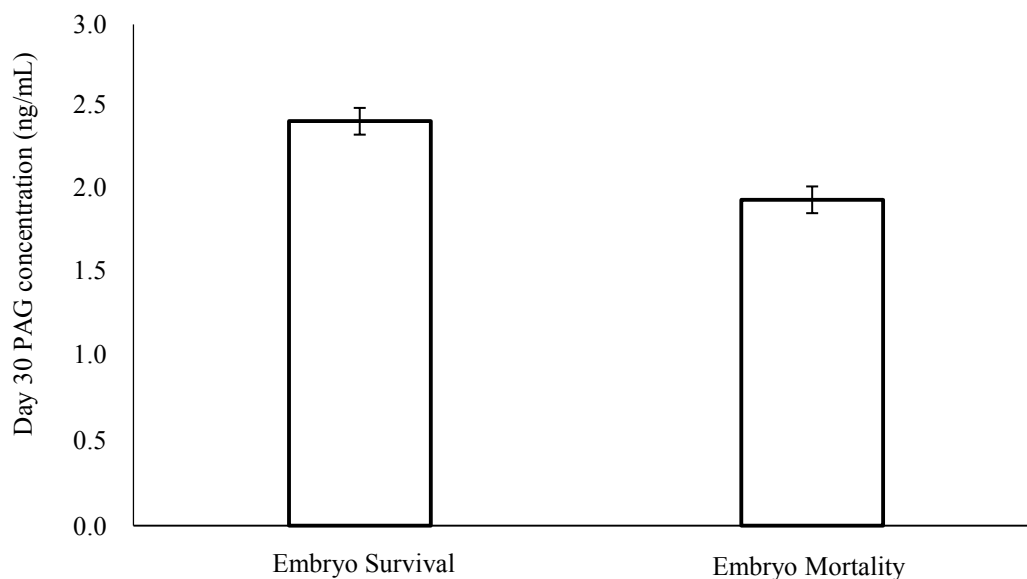

Figure 1. Serum concentrations of pregnancy-associated glycoproteins (PAGs) in heifers that had a embryonic heartbeat on day 30 and maintained pregnancy (Embryo survival; n = 406) or did not maintain pregnancy (Embryo mortality; n = 21). Heifers that had embryo mortality between gestation days 30 and 65 had significantly less PAG serum concentrations than heifers that maintained pregnancy.
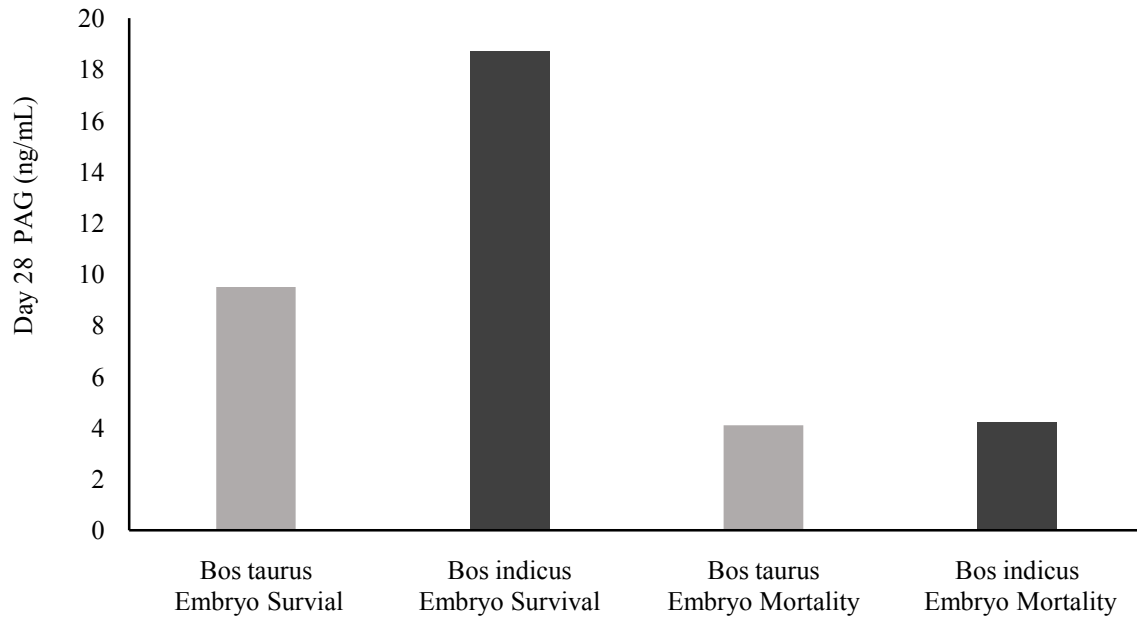
Figure 2. Serum concentrations of pregnancy-associated glycoproteins (PAGs) in samples collected on day 28 of gestation from pregnant *Bos taurus* cows and *Bos indicus* cows with a viable embryo based on fetal heartbeat. Cows were then categorized into whether they maintained pregnancy until day 72 (*Bos Taurus;* n = 1416) or day 100 (*Bos indicus;* n = 1365) of gestation (Embryonic survival) or embryonic mortality (between days 29 to 72 or 100; *Bos Taurus;* n = 171; *Bos indicus;* n = 213). Beef cows that experienced late embryonic mortality had decreased (P < 0.05) circulating concentrations of PAGs on day 28 compared to cows that maintained an embryo.

A multitude of factors have may affect circulating PAG concentrations including subspecies, parity and sire. Despite a correlation between embryonic size and placental size, results indicate no significant relationship between PAG concentration and embryo size during early gestation (Pohler *et al.*, 2014). Lack of correlation indicates that the decrease in PAG concentration in cows experiencing late embryonic mortality is indicative of impaired placental or endometrial function, not slow embryonic growth. In addition*, Bos indicus* cattle tend to have greater circulating PAG concentrations compared to *Bos taurus* cattle (Pohler *et al.*, 2016b). Any *Bos indicus* influence in the genetic base will increase PAG concentrations over a straightbred *Bos taurus* cow (Mercadante *et al.*, 2013). Others have reported that profiles of circulating PAG are similar between subspecies although *Bos indicus* cows may have a smaller relative increase in the weeks preceding parturition (Sousa *et al.*, 2003). High producing dairy cows exhibit a significant negative correlation between milk production and PAG concentration. In a study by Lopez-Gatius *et al.* (2007), each 1 kg increase in milk resulted in a decrease in PAG of 0.08 to 0.1 ng/ml. Perhaps even more interesting are the effects of parity and sire on PAG concentrations. Heifers have consistently been reported to have the greatest PAG concentration and as parity increases, mean PAG concentration subsequently decreased in a somewhat linear fashion until the 3 or 4th parity (Lobago *et al.*, 2009; Ricci *et al.*, 2015; Pohler *et al.*, 2016b). Recent work has examined sire differences on PAG concentration due to the paternal influence over trophoblast and placental development. Preliminary evidence suggests that pregnancies produced by bulls accounting for decreased rates of late EM may exhibit increased PAG concentrations compared to bulls that result in increased rates of late EM (Pohler *et al.*, 2016b).

Estrus expression at the time of insemination or prior to embryo transfer has been directly correlated with pregnancy success in both beef and dairy cattle (Perry *et al.*, 2005; Pereira *et al.*, 2016). In a recent study (Pereira *et al.*, 2016), lactating dairy cows undergoing TAI or embryo transfer had increased fertility and decreased embryonic morality if they exhibited estrus versus those that did not exhibit estrus. Furthermore, lactating dairy cows with pregnancy loss had decreased circulating concentrations of PAGs early in gestation (Pohler *et al.*, 2016a), similar to the current study. In a study with postpartum beef cows, there was an increase in PAG concentrations on day 28 of gestation when comparing estrotech patch scores at TAI (day 0). Surprisingly, previous work has not demonstrated an association with pre/postovulatory estradiol or progesterone production with PAG production early in gestation (Pohler *et al.*, 2013). Thus, these data indicate that cows which exhibit estrus and conceive have increased circulating concentrations of PAGs on d 28 and increased likelihood of pregnancy success compared with pregnant cows that did not express estrus at TAI. Future experiments are needed in this area to truly understand this relationship and potential mechanism that is underlying this increase in PAG production.

Conclusions

Although numerous detection methods can

accurately diagnosis pregnancy in cattle, PAGs and pregnancy specific miRNA are biomarkers that can be used before day 30 of gestation and may be useful in predicting embryonic mortality. Although pregnancy diagnosis may be possible earlier in gestation, benefits may be mitigated by high incidence of embryonic mortality after day 24.

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