Using Pregnancy Associated Glycoproteins (PAGs) to understand and enhance fertility in cattle

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Key Points:

· Pregnancy diagnosis from a blood sample enables the detection of nonpregnant cows earlier than rectal palpation after insemination or embryo transfer

· Pregnancy diagnosis using PAGs is an efficient method that is based on detecting the presence of a pregnancy-specific protein

· PAG testing is commercially available in both blood and milk

· PAG testing may also provide a useful tool for detection of pregnancies that have a high probability of undergoing late embryonic mortality

Introduction:

Reproductive failure is one of the most substantial barriers to profitability in beef herds. Management issues, cow infertility, bull infertility, heat stress and embryonic mortality are all contributing factors to reproductive inefficiency. The brazilian beef herd has over 70 million cows and embryonic mortality represents a lost of almost 4 billion reais/years[(1)]. Brazil has been at the forefront of reproductive technology adoption, including fixed time artificial insemination (FTAI), estrus synchronization (ES), in vitro embryo production (IVP), pregnancy testing and chemical based pregnancy testing (PAGs), in order to increase genetic progress of the national beef herd. Brazilian semen sales of beef sires increased from 3.3 million units in 1993 to 11.9 million units in 2011[(2)]. Between 11-12% of the total beef herd is being inseminated through AI which equates to 5.5 million cows, this is the largest proportion in the world[(3)]. Although these technologies have numerous benefits, producers must dedicate time and labor resources to successfully implement them. Traditional estrus detection protocols require monitoring twice

daily for extended periods of time and excludes animals that do not show estrus from insemination. Fixed time artificial insemination and ES protocols designed to be used in timed synchronization have been used with similar results to that of estrus detection AI with less input. Subsequently, the adoption of FTAI protocols has increased among producers. The question is: "How can we as producers further increase pregnancy rates to a single insemination or increase reproductive efficiency?" The answer to that question is not so easily resolved and it has prompted further investigations of how to increase dominant follicle maturity, increase oocyte competence, improve the uterine environment and promote placental health, to name a few. Our group has chosen to explore the use of PAGs (chemical based pregnancy testing) to increase reproductive efficiency in cattle.

Maternal circulating of PAGs as tools for reproductive management in cattle

PAGs and pregnancy establishment:

Members of the modern PAG family are detectable in the maternal circulation by multiple tests (e.g. RIA and ELISA) starting soon after the time of binucleate cell formation (day 19-20 of gestation)[6] until a few weeks after parturition (**Figure 1**).[7,8] Circulating concentrations of bovine PAGs can be influenced by a number of factors including breed, weight, parity status of the dam, fetal sex, fetal number, and fetal birth weight, along with pregnancy stage and status.[9,10] However, the role that PAGs play during gestation remains undefined.

To date there have been no clear functions related to modern PAGs; however, PAGs have been shown to inhibit different immune cells, in vitro, and may camouflage fetal/placental antigens from the immune system.[23]A majority of the work on PAGs has focused on the development of a reliable tool for diagnosing pregnancy in multiple ruminant species including cattle, sheep, goats, buffalo, bison, moose, and elk.[11] PAGs are unique compared to other

biochemical methods of pregnancy detection in cattle because these proteins are pregnancy specific. PAG1 (also known as pregnancy specific protein B; PSPB) has been the primary PAG of most interest in relation to early pregnancy diagnosis because of the ability to detect PAG1 in the maternal circulation throughout gestation.[7,12] However, Greenet et al., [13] highlighted two disadvantages in using PAG1 for pregnancy detection: 1) pregnancy diagnoses in the first month of pregnancy could be compromised due to the low and variable circulating concentrations of PAG1, and 2) the long half-life of these proteins (~8 days) in the maternal circulation after partition or fetal loss. Due to these concerns, there has been interest in detecting other PAGs for pregnancy detection. Green et al., [8] reported the establishment of an ELISA based test for early pregnancy PAGs with a relatively short half-life (4.3 days). It has also been shown that PAG concentrations first significantly increase in circulating around day 24 of gestation followed by a transient rise out to partition in cattle [8, 14] which is similar to that of other small ruminants. In the preceding study, PAGs were detected in all cattle by d 28 of gestation, PAG concentrations peaked around the time of parturition. After parturition PAGs were undetectable by eight weeks postpartum in 38 of the 40 cows, thus concluding that choosing different PAGs helps overcome the persistence of PAG immunoreactivity far into the postpartum period. In similar studies, after induced embryonic mortality, the half- life of circulating concentrations of PAGs was determined to be 35.8 ±21.9 h.[14] These differences in PAGs half-live are presumably a result of distinct forms of the PAG family present earlier in gestation compared to term or a result of different clearance mechanisms between early and late pregnancy.

There are currently three commercial PAG testing platforms available for use, 1) BioPRYN (BioTracking, LLC, Moscow, ID), 2) DG29 (Conception Animal Reproduction Technologies, Beaumount, QC), and IDEXX Bovine Pregnancy Test (IDEXX Laboratories, Inc.

Westbrook, ME). Current PAG assays have been documented to accurately diagnose pregnancy in cattle with an average accuracy ranging from 93 to 96% in both blood and milk. [8, 14-20]

PAGs as a predictor of late embryonic mortality and as a biochemical marker for placental function:

In cattle, the incidence of late embryonic/early fetal loss around the time of embryo uterine attachment is approximately 4 to 10%.[17, 23-27] Late embryonic mortality may have a more significant economic impact due to the delay in conception date resulting in more variation in calf birth dates and weights within a single calving season.

The mechanisms associated with reproductive loss around the time of placentation are unknown, but may be associated with inadequate placental development or function. Along with the ability to use PAG assays as tools for pregnancy-detection, PAGs may also serve as a marker for monitoring embryonic/fetal viability along with placental function. For example, beef cows that successfully carried a pregnancy past day 72 of gestation had higher circulating concentrations of PAGs on day 28 compared to cows that exhibited late embryonic/fetal mortality between day 28 to 72 (using a sandwich ELISA).[17, 26] In the preceding studies, all cows had an embryo with a heartbeat on day 28 of gestation; however, cows that experienced late embryonic/fetal mortality after day 28 and before day 72 had decreased circulating concentrations of PAGs on day 28. Similar data have been reported in dairy cows[23, 25, 28] and sheep[27] in which circulating concentrations of PAGs were higher or lower in animals that maintained or lost a pregnancy, respectively. However, Ricci et al., [17] reported that PAGs were not predictive of late embryonic mortality in dairy cattle. The preceding discrepancy in the efficacy of utilizing circulating concentration of PAGs on day 28 to 30 to predict late embryonic mortality in cattle may be explained by the specific PAG assay that was employed. There seems to be no correlation between embryonic size (crown rump length), embryonic width or

embryonic volume at day 35 or 56 of gestation in beef cattle suggesting that these lower concentrations of PAGs in the maternal circulation are not purely reflective of a smaller embryo.

A recent study demonstrated that bPAGs were 96% accurate in diagnosing pregnancy in *Bos indicus* cows. As with previous studies using the sandwich ELISA in *Bos taurus* cows, this assay could accurately predict late embryo mortality based on decreased bPAG concentration at day 28 of gestation (**Figure 2 and 3**). However bPAG concentration cannot account for pregnancy loss that occurs after day 45 due to ovarian failure or other physiological condition [(29)].

Concentrations of bPAG early in gestation are higher in *Bos indicu*s cows that *Bos taurus* cows at similar stages although the mechanism is unknown (**Figure 4**)[(29, 30)]. PAG concentration of *Bos indicus* cows that maintained pregnancy were 8 to 10 ng/mL higher than *Bos taurus* cows that maintained pregnancy however in both subspecies cows that suffered embryonic mortality had comparable levels of serum PAGs. Heifers also have higher PAG concentration than multiparous cows that is independent of body weight and blood volume[(29)].

Pohler et al[(29)] also examined the effect of estrus heat strength on bPAG concentration. Heat strength was measured with the application of an Estrotect patch where increased mounting by other animals would change the color of the patch. Animals that had been mounted multiple times would be given higher patch scores than animals that had not been mounted. Cows that exhibited stronger estrus also had higher bPAG concentrations. Those with a patch score of 3 or 4 had a statistically higher concentration than patch scores of 1 and 2, which correlated with higher pregnancy rates in the cows with increased bPAG expression (**Figure 5**).

Influence of sire fertility on PAGs maternal circulation

Pregnancy status and stage, breed, parity of dam, fetal sex and number, fetal birth weight, placental weight, sire, and many more have been shown to be associated to some degree with

bPAG concentrations[9,10,14]. Limited data have been reported on sire effects on bPAG concentrations early in gestation; however, based on the large influence that the sire plays in placental development we were interested in examining this relationship. Overall, from our previous studies, we saw no relationship between circulating concentrations of bPAGs and sire fertility, but there was a large amount of variation across sires and bPAG production (**Figure 6**)[29]. In addition, of eight sires tested three accounted for 70% of the late embryonic mortality reported in the subset of cows. Surprisingly, after removing from the analysis all the cows that underwent late embryonic mortality after day 28, those three sires exhibited significantly decreased circulating concentrations of bPAG compared to the other five sires in the study (**Figure 7**). Taken together these data suggest that the sire does influence BNC products, such as bPAGs. Indeed, circulating amounts of bPAG may serve as a novel tool for identifying low fertility sires.

Summary

Overall, using a biochemical marker such as PAG in ruminant ungulates may provide a powerful technique for a producer for identifying pregnant animals along with selecting cows that are most likely to experience embryonic/fetal loss thus increasing reproductive efficiency. As we saw that multiple factors such as parity status and sire were shown to affect circulating concentrations of bPAG at day 28 the circulating amounts of bPAG may serve as a novel tool for identifying low fertility sires. Our current working hypothesis is that PAGs released into the maternal circulation provide a useful tool for not only identifying pregnancy status, but also viablility of the developing placenta and embryo/fetus (**Figure 8**).

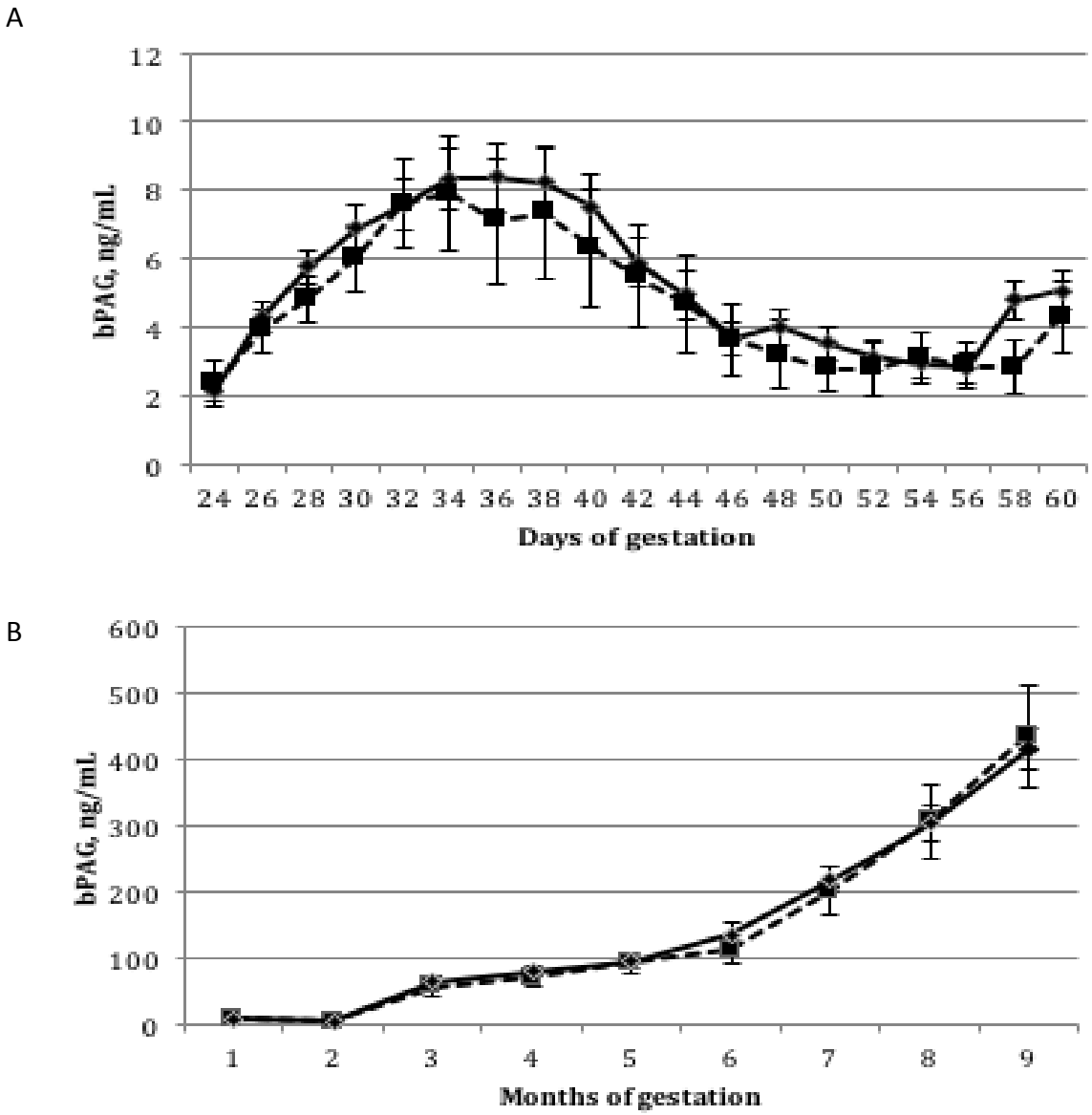

Figure 1. Circulating concentrations of PAGs during gestation in pregnant beef cows. Panel A represents the first 60 days of gestation and Panel B the entire 9 months of gestation. Interestingly, the drop in circulating PAG on about day 38 to 40 is repeatable in any breed or type of cattle.

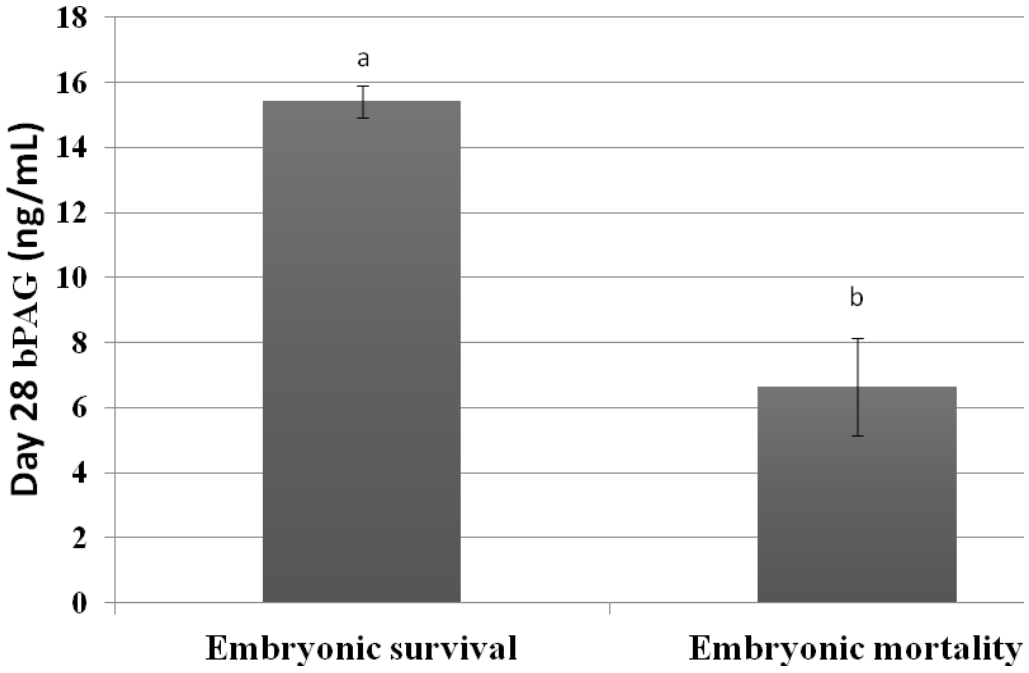

Figure 2. Serum concentrations of bPAGs (mean±SEM) in postpartum Nelore beef cows which received TAI on day 0 and had a viable embryo on day 28 of gestation (n=803) and either maintained (embryonic survival; n=714) or experienced embryonic mortality (n=89) by day 100. Nelore cows that experienced late embryonic mortality by day 100 of gestation had decreased ($P<0.05$) circulating concentrations of bPAGs on day 28 compared to cows that maintained an embryo until day 100.

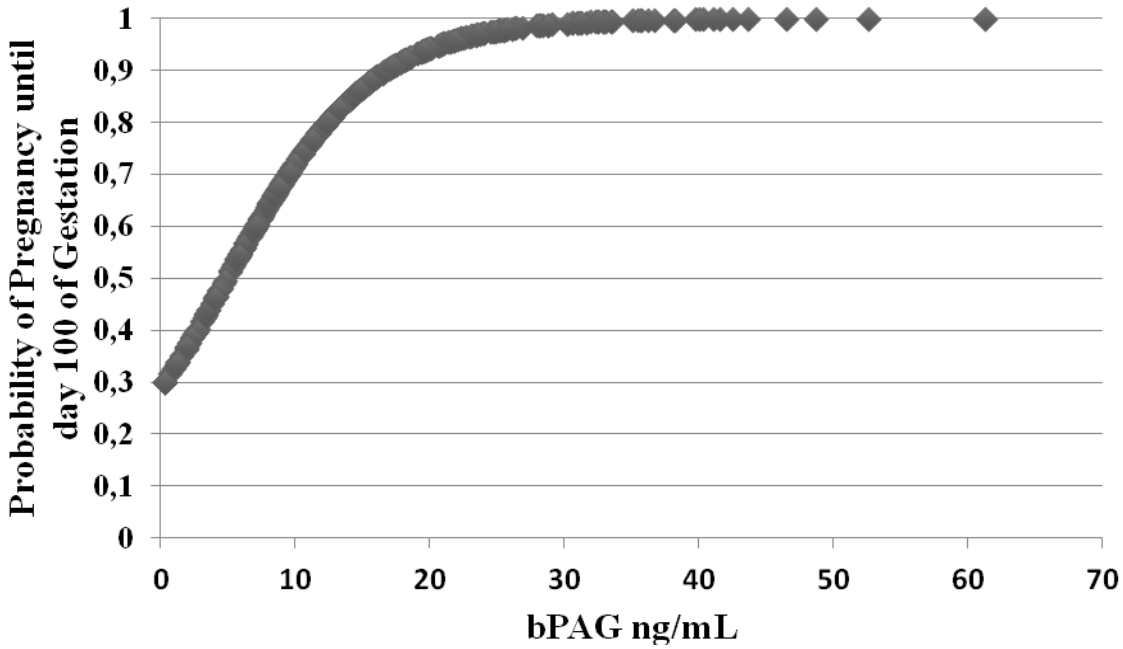

Figure 3. Probability of pregnancy maintenance following TAI between day 28 to 100 of gestation based on day 28 serum concentrations of bPAGs (n=803). Increased serum concentrations of bPAGs on day 28 significantly increased (P<0.05) the probability of pregnancy maintenance until day 100 of gestation in Nelore beef cows following TAI.

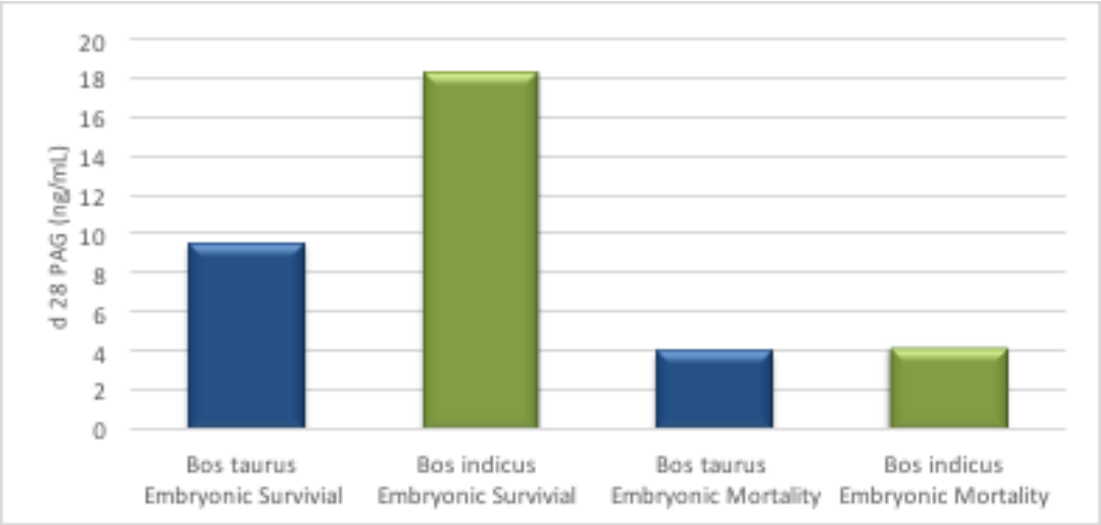

Figure 4. Serum concentrations of PAGs in samples collected on day 28 of gestation from pregnant *Bos taurus* cows and *Bos indicus* cows with a viable embryo based on fetal heartbeat. Cows were then divided into whether they maintained pregnancy until day 72 (*Bos taurus*) or day 100 (*Bos indicus*) of gestation (Embryonic survival) or embryonic mortality (between day 29-72 or 100). Beef cows that experienced late embryonic mortality had decreased (P<0.05) circulating concentrations of bPAGs on day 28 compared to cows that maintained an embryo. Modified from Pohler, 2015 & Pohler.[(11)]

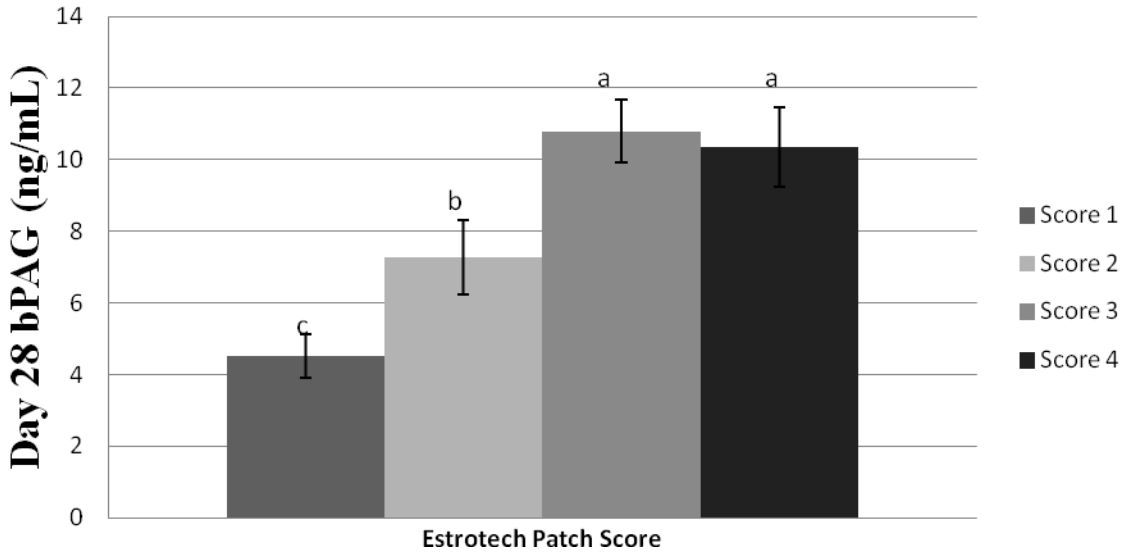

Figure 5. Serum concentrations of pregnancy associated glycoproteins of cows that exhibited different estrus intensities. Cows that exhibited stronger estrus by means of Estrotect heat dectors patch score had higher bPAG concentration on day 28.

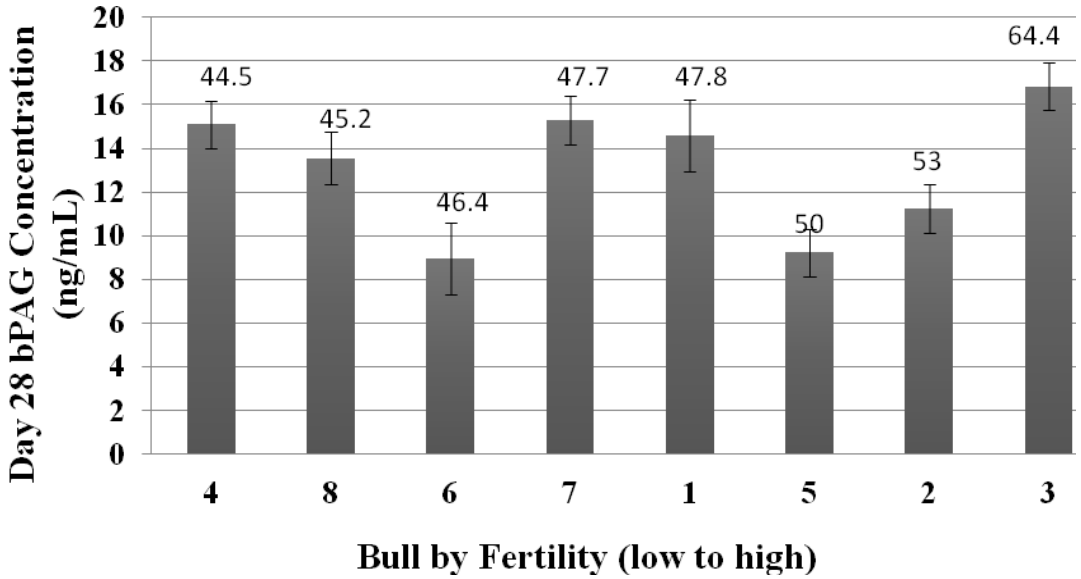

Figure 6: Serum concentrations of bPAGs on day 28 of gestation from cows with pregnancies sired by sires 1 to 8. Although there was variation in pregnancy rate to TAI among sires (44 to 64%), there was no linear relationship between pregnancy rate by sire and circulating concentrations of bPAGs. However, there were significant differences in circulating concentrations of bPAGs among sires.

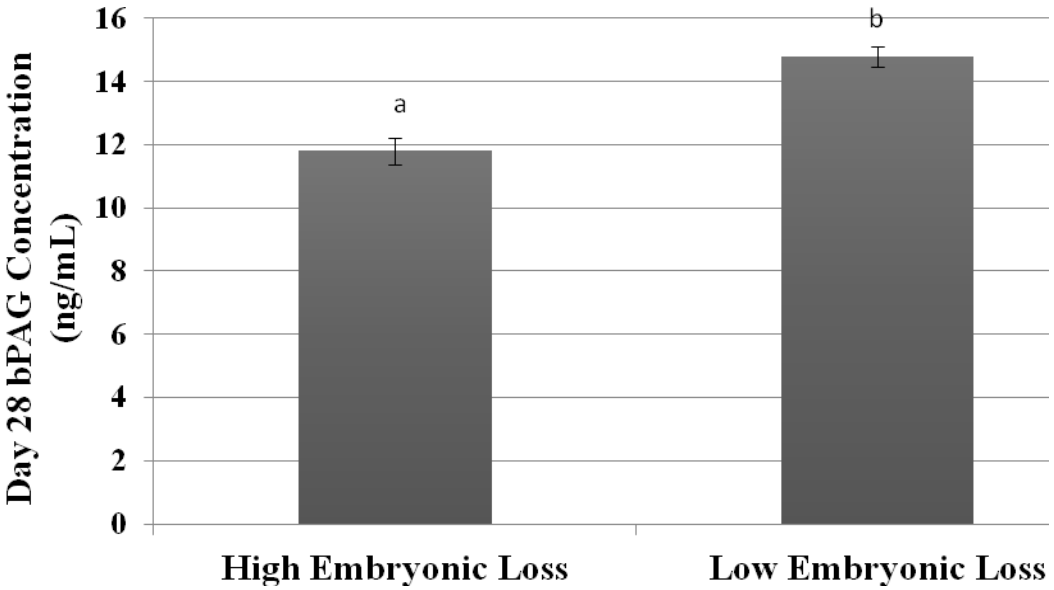

Figure 7. Serum concentrations of bPAGs on day 28 of gestation between sires that resulted in high embryonic loss and sires that resulted in low embryonic loss. After removing all cows that lost a pregnancy after day 28 form the data set, the sires with the highest incidence of late embryonic mortality also were the sires with pregnancies that produced significantly ($P < 0.05$) lower maternal circulating concentrations of bPAGs on day 28 of gestation compared to the remaining sires that had pregnancies having low embryonic mortality.

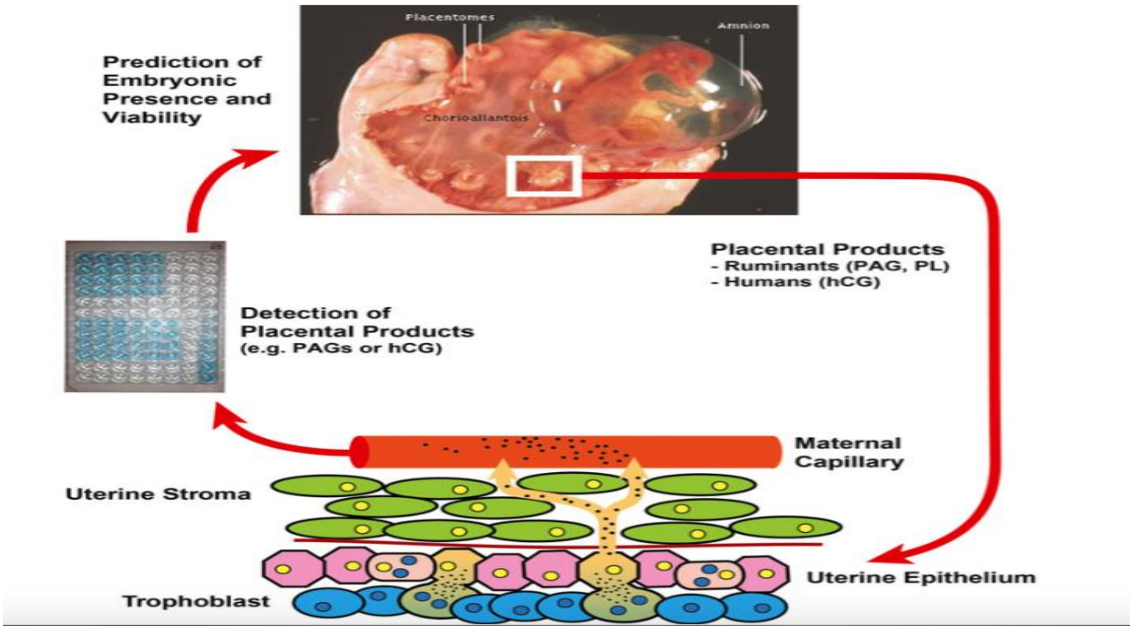

Figure 8: The figure shows production of pregnancy associated glycoproteins (PAGs) and placental lactogen (PL) by binucleated trophoblast cells (BNC) within a placentome of the ruminant placenta. BNCs fuse with the uterine epithelial cells to form trinucleated cells, and PAGs and PL subsequently enter the maternal circulation. The working hypothesis is that placental products can be used to monitor conceptus presence and well being. Figure modified from Pohler et al., 2015.

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