BOVINE PREGNANCY ASSOCIATED GLYCOPROTEINS IN BEEF CATTLE: EFFECTS OF EMBRYO SIZE AND VARIOUS METHODS OF HANDLING OF PLASMA AND SERUM SAMPLES

A Thesis

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ABSTRACT

Accurate assessment of pregnancy associated glycoproteins (PAG) in biological samples is fundamental for early pregnancy diagnosis and prediction of embryonic mortality in cattle. Two experiments were conducted to determine the effects of embryo size and various methods of handling of plasma and serum samples on PAG in beef cattle. The objective of experiment 1 was to determine a relationship between embryonic size and growth throughout gestation and circulating PAG concentrations. A total of 137 multiparous beef cows at three different locations were used for this experiment. Embryonic crown to rump length (CRL) was measured via transrectal ultrasound and PAG concentrations were determined using an in-house enzyme-linked immunosorbent assay (ELISA). Data were analyzed using PROC CORR (SAS 9.4). Birthweight was not significantly correlated with PAG concentrations on days 28 and 67 at Location 1 (r=-0.24; *P*=0.12 and r=-0.11; *P*=0.47, respectively). PAG on day 42 was associated with day 70 CRL at Location 3 (r=0.43; *P*= 0.04); however, no other CRL measurements were significantly associated with PAG concentrations. Few significant correlations between embryo-fetal growth and PAG concentrations were only detected at Location 3; however, the strength of the relationship lacked statistical power. In conclusion, PAG concentrations throughout different stages of gestation do not appear to be associated with embryo size or development. The objective of experiment 2 was to identify the effect of plasma or serum samples, time to centrifuge, repeated freeze-thaw cycles and antibody handling on PAG concentrations. Plasma and serum samples were collected

from 2 pregnant and 2 open multiparous beef cows and evenly distributed to be centrifuged on the same day or one day post-collection. Samples were submitted to one, two and three freeze-thaw cycles, a no-thaw sample (pre-freezing), and an intact sample (no freeze-thaw cycles)were analyzed with an in-house ELISA. Data were analyzed using PROC GLM (SAS 9.4). A decrease in PAG concentrations was detected for serum compared to plasma ($P<0.0001$); however, plasma PAG concentration was not affected by multiple freeze-thaw cycles ($P= 0.19$) although serum concentrations were ($P= 0.03$). Interestingly, centrifugation day did not alter PAG concentrations in either plasma or serum ($P= 0.50$ and $P= 0.59$, respectively), nor did the utilization of frequently used monoclonal antibodies versus unfrequently used monoclonal antibodies ($P= 0.89$). In conclusion, plasma appears to be a more reliable source for detecting PAG concentrations.

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TABLE OF CONTENTS

Page

ABSTRACT ii

ACKNOWLEDGEMENTS iv

CONTRIBUTORS AND FUNDING SOURCES v

TABLE OF CONTENTS vi

1. INTRODUCTION 1

2. LITERATURE REVIEW 4

2.1. Pregnancy associated glycoproteins 4
2.2. Enzyme-linked immunosorbent assay 9
2.3. Antibody dependence 12
2.4. References 14

3. EFFECT OF EMBRYO SIZE ON BOVINE PREGNANCY ASSOCIATED GLYCOPROTEINS THROUGHOUT DIFFERENT STAGES OF GESTATION 27

3.1. Synopsis 27
3.2. Introduction 28
3.3. Materials and methods 31
3.3.1. Locations 31
3.3.2. Blood sampling and pregnancy associated glycoproteins 32
3.3.3. Pregnancy diagnosis and embryo morphometry 33
3.3.4. Statistical analysis 34
3.4. Results 35
3.4.1. Location 1 35
3.4.2. Location 2 35
3.4.3. Location 3 35
3.5. Discussion 36
3.6. Conclusion 41
3.7. References 42

4. EFFECT OF VARIOUS METHODS OF HANDLING OF PLASMA AND SERUM SAMPLES ON BOVINE PREGNANCY ASSOCIATED GLYCOPROTEINS 53

4.1. Synopsis 53
4.2. Introduction 54
4.3. Materials and methods 56
4.3.1. Experiment design 56
4.3.2. Blood sampling and centrifugation effect 57
4.3.3. Thawing and freezing effect 57
4.3.4. Monoclonal antibody effect 58
4.3.5. Pregnancy associated glycoprotein assay 58
4.3.6. Statistical analysis 58
4.4. Results 59
4.5. Discussion 60
4.6. Conclusion 66
4.7. References 67

5. CONCLUSIONS 74

APPENDIX A TABLES 76

APPENDIX B FIGURES 80

1. INTRODUCTION

Embryonic mortality and pregnancy loss are major challenges for livestock production that results in substantial economic loss. In the United States, reproductive failure can annually cost an estimated $600 million and $1 billion for the beef and dairy industries, respectively (NAHMS, 2020). In general, pregnancy loss can vary between 3 to 42% depending on gestational stage (Vasconcelos et al., 1997; Santos et al., 2004; Diskin and Morris, 2008; Ribeiro et al., 2013; Gatea et al., 2018). Pregnancy loss can be further divided into early embryonic mortality (<28 day of gestation) and late embryonic or fetal mortality (from day 28 to approximately day 45 of gestation) (Hubbert et al., 1972; Pohler et al., 2013; Gatea et al., 2018). Pregnancy-specific markers such as pregnancy associated glycoproteins (PAGs) can be used to better understand and predict embryonic mortality (Tonhatti, Lobo, and Oliveira, 1999).

Ruminant ungulates produce PAGs; which are proteins exocytose from binucleate trophoblast cells (BNCs) into the maternal circulation after implantation (around day 19 to 21 of gestation) (Wooding, 1983; Zoli et al., 1992). Although the specific functionality is yet to be determined, many suggest an influence in control of growth factors associated with placental-uterine interface, maternal immune system, and placental function and/or efficiency (Hoeben et al., 1999; Pohler et al., 2013; Wallace, Pohler, Smith, and Green, 2015). Diagnosing pregnancy in cattle by measuring maternal circulating PAGs in blood or milk samples is 98-99% accurate in pregnancy detection between days 28 and 30 of gestation (Green et al., 2005; Pohler et al., 2013; Wallace,

Pohler, Smith, and Green, 2015). Several studies have identified that a decrease in maternal circulating PAG concentrations is directly correlated to reduced probabilities of maintaining pregnancy until term (Thompson et al., 2010; Breukelman et al., 2012; Pohler et al., 2013; Pohler et al., 2016a; Pohler et al., 2016b; Gatea et al., 2018). Many factors can influence PAG concentrations like *in vitro* produce (IVP) and cloned-derive embryos (Farin, Piedrahita, and Farin, 2006; Constant et al., 2011; Pohler et al., 2016a). Fetuses derived from these pregnancies are prone to abnormalities like Large Offspring Syndrome (LOS) which is associated with placental deficiency (Heyman et al., 2004; Lee et al., 2004; Constant et al., 2006). As previously stated, PAGs may be markers for placental efficiency, suggesting a relationship between embryo size, maternal circulating PAG concentrations and placental functionality.

Differences in PAG concentrations may also be affected by sample type and handling. Studies utilizing serum samples detected a correlation between embryonic mortality and maternal circulating PAGs (Breukelman et al., 2012; Pohler et al., 2016b); while Ricci et al. (2015) failed to detect an association with plasma samples. Pregnancy diagnosis does not seem to be altered by sample type although differences in proteins levels have been found between serum and plasma samples (Kaneko, Harvey, and Bruss, 2008; Thompson et al., 2010; Commun et al., 2016); suggesting that embryonic mortality may be more sensitive to changes in PAG levels. Moreover, multiple freeze-thaw cycles, which occur in a laboratory setting, can also alter proteome integrity in serum and plasma samples by denaturation and degradation (Mitchell et al., 2005; Lee, Kim, and Shin, 2015; Lugos, Polit, and Damen, 2018). Therefore, the goal of this

research is to 1) characterize the relationship between embryo size and PAG concentrations at different stages of gestation and 2) determine if plasma and serum PAG concentrations are altered by repeated freeze-thaw cycles, centrifugation times and frequency of antibody usage, when contamination is avoided.

2. LITERATURE REVIEW

2.1. Pregnancy associated glycoproteins

Over the past few decades, many pregnancy-specific markers have been identified with one of the first being pregnancy-specific protein B otherwise known as pregnancy associated glycoprotein 1 (PAG 1) in cattle (Butler et al., 1982). Pregnancy associated glycoproteins genes can be found in species within the Artiodactyla order (i.e., ruminant ungulate species such as cattle) and there have been over 22 transcribed genes and variants identified specifically in the bovine PAG gene family (Telugu, Walker, and Green, 2009). Bovine PAG genes can be separated into two main groups according to its transcription site: "ancient" and "modern" PAG (Green et al., 2000; Wooding, Roberts, and Green, 2005), with PAG-1 belonging to the latter group. Ancient PAG genes are detectable in the filamentous conceptus, throughout pregnancy they are usually found in mononucleated trophoblast cells although they can also be expressed by giant binucleate trophoblast cells (BNCs). In contrast, modern PAGs and placental lactogen are predominantly produced by BNCs (Wooding, 1981; Zoli et al., 1992). By day 19 to 21 of gestation, BNCs are thought to be derived from merging trophoblast cells; however, such results have only been confirmed in ovine (Wooding, 1983; Seo, Bazer, Burghardt, and Johnson, 2019). The BNCs migrate from the trophectoderm through microvillar junctions and fuse with the uterine epithelium to release contents, such as PAGs, into the maternal circulation via exocytosis (Garbayo et al., 2000; Wallace, Pohler, Smith, and Green, 2015). This process is achieved by a more direct

fetal-maternal interaction found in synepitheliochorial type of placenta animals (e.g. ruminants) where there is fusion between the uterine epithelium and trophoblast cells; as opposed to its epitheliochorial counterparts where the uterine epithelium does not invade (e.g., pig and horses) (Wallace, Pohler, Smith, and Green, 2015).

There is a noticeable increase in PAG concentrations found in plasma during mid- and late gestation, specifically nearing parturition (Sasser et al., 1986; Green et al., 2005). The exact mechanisms for this increase in PAGs remains unclear, but studies imply an accumulation in different tissues that are being subsequently released close to parturition (Sasser et al., 1986; Zoli, Ectors, and Beckers, 1990); Green et al., 2005). Additionally, presence of PAG-like immunoreactivity has been found in bulls and in virgin heifers, suggesting an accessory source of PAGs yet to be discovered (Zoli et al., 1992). The exact physiological role of PAGs has yet to be determined; but an immune, luteotropic, placental-uterine interface govern by growth factors, along with placental function and/or efficacy has been suggested (Austin et al., 1999; Hoeben et al., 1999; Pohler et al., 2013; Wallace, Pohler, Smith, and Green, 2015). Pregnancy associated glycoproteins can be detected as early as 22 to 24 days of gestation in maternal circulation (Zoli et al., 1992; Xie et al., 1997; Green et al., 2000; Green et al., 2005), suggesting the potential to be used as a biomarker for early pregnancy detection.

With placental attachment occurring around day 17 of gestation in cattle (Roberts, Xie, and Mathialagan, 1996), concentrations of PAGs increase by approximately 10% in pregnant lactating dairy cows (Middleton and Pursley, 2019). A difference in maternal circulating PAG concentrations can be detected between pregnant

cows and heifers at day 24 of gestation; with some studies finding greater levels in pregnant heifers (Arnold et al., 2012; Oliveira Filho et al., 2020) while others indicate pregnant cows (Reese et al., 2018). Apart from total concentration levels, Arnold et al. (2012) detected a substantial increase in PAG serum levels earlier in gestation in pregnant dairy heifers than pregnant dairy cows. Pregnant animals had increased maternal circulating PAG concentrations that non-pregnant animals on day 24 of gestation; however, there was no significant difference in animals that maintained pregnancy versus those that experienced embryonic mortality (Reese et al., 2018). Suggested differences are attributed to the irregularity in PAG concentrations found between day 24 and 30 of gestation along with the wide variability of genes detected solely on the PAG family and the antibody dependence inherent to each PAG gene (Breukelman et al., 2012; Gatea et al., 2018). Antibody dependence will be further explained in a subsequent part of the literature review. Overall findings suggest that circulating PAG concentrations on day 24 could be used as a marker to detect pregnancy, however, it may overlook early embryonic mortality.

Contrary to maternal circulating PAG concentrations on day 24 of gestation, concentrations on day 28 provide a more accurate pregnancy diagnosis and marker for embryonic morality. Compared to transrectal ultrasonography which can be performed as early as day 26 to 29 of gestation (Curran, Pierson, and Ginther, 1986; Kastelic, Curran, Pierson , and Ginther, 1988), circulating PAG concentrations on day 28 have an accuracy between 98-99% of detecting pregnant vs non-pregnant cows (Pohler et al., 2013; Pohler et al., 2016b). Currently PAGs can be detected by commercial tests

(Romano and Larson, 2010; Speckhart et al., 2018) using blood or milk samples such as BioPRYN, IDEXX, and Genex (BioPRYN; BioTracking LLC, Moscow, ID; IDEXX Bovine Pregnancy Test; IDEXX Laboratories Inc., Westbrook, ME; and D29 Blood Pregnancy Test; Genex Cooperative Inc., Shawano, WI). The remaining 1-2% of accuracy that commercial kits fail to detect is attributed to embryonic mortality occurring later in gestation and not a failure of the procedure *per se*.

Circulating concentrations of PAGs can be used as predictors of embryonic mortality, specifically late embryonic mortality (after day 28 of gestation) (Thompson et al., 2010; Breukelman et al., 2012). Pregnancy associated glycoproteins detected in serum samples between days 25 and 32 of gestation of dairy cows subjected to fixed/timed embryo transfer (FTET) were influenced by pregnancy loss; with decreased levels of PAGs associated with greater embryonic death between days 26 and 100 of gestation (Breukelman et al., 2012). Likewise, PAG concentrations in serum samples on day 31 of gestation below 1.4 ng/mL in fixed-timed artificial insemination (FTAI) and 1.85 ng/mL in FTET were 95% accurate in predicting late embryonic mortality in dairy cattle (Pohler et al., 2016a). Similar accuracy results with FTAI and late embryonic mortality were found on Nelore beef cows, with a cutoff of less than 0.72 ng/mL on day 28 of gestation (Pohler et al., 2016b). Reese et al. (2019) had similar results when comparing sub fertile heifers versus high fertility heifers using a serial embryo transfer model. Heifers with decreased concentrations of PAGs on day 28 of gestation in the circulation experienced greater pregnancy losses regardless of fertility group. Although several studies found a relationship between embryo survival and PAG concentrations,

Ricci et al. (2015) failed to detect such levels of accuracy in predicting embryonic mortality when using a commercial kit as opposed to an in-house sandwiched ELISA performed by other researchers who detected a correlation between both factors. Overall, the combination of studies suggests that an increased circulating PAG concentrations early in gestation is directly correlated to decreased likelihood of embryonic losses; however, further research must be completed to better understand differences in results (Szenci et al., 2000; Thompson et al., 2010; Pohler et al., 2014). An insight on differences caused by specific usage of commercial kits or in-house ELISAs will be addressed later.

Pregnancy associated glycoprotein concentrations in the maternal circulation can be influenced by multiple factors such as: subspecies, parity, sire, day of gestations, and fetal sex (Patel et al., 1997; Mercadante et al., 2013; Ricci et al., 2015; Pohler et al., 2016a; Franco et al., 2018). Additionally, it has been suggested that PAG concentrations differ between transferred embryos produced by *in vitro* fertilization (IVF) and pregnancies obtained from natural mating or artificial insemination (AI) (Constant et al., 2011; Pohler et al., 2016a; Gatea et al., 2018). Differences can be attributed to developmental abnormalities derived from *in vitro* produced (IVP) embryos (Farin and Farin, 1995; Farin, Piedrahita, and Farin, 2006); however, others have failed to observe any differences between IVP and *in vivo* produced embryos (Breukelman et al., 2005; Breukelman et al., 2012). Cloned bovine embryos have also associated with altered circulating PAG concentrations which can be attributed to placental abnormalities (Chavatte-Palmer et al., 2006; Constant et al., 2011). Interestingly, ovarian physiological

characteristics prior to embryonic development, e.g. size of ovulatory follicle at time of AI, does not have an effect on PAG concentrations during gestation (day 28) (Pohler et al., 2013). The same study was unable to find an influence of embryo stage and/or quality on PAG concentrations. Genotype or subspecies can also alter maternal circulating PAG concentrations as suggested by Mercadante et al. (2013) in which *Bos indicus*-influenced cows had greater serum PAG concentrations; however, such study failed to detect repeatability in observations. Moreover, *Bos indicus* influenced embryos possess increased concentrations of PAGs as well (Fontes et al., 2019). Franco et al. (2018) also detected an influence by sire in maternal circulating PAG concentrations with *Bos taurus* sired pregnancies having greater concentrations than *Bos indicus* sires when Nelore cows (*Bos indicus*) were subjected to AI. However, consistency within studies was not found as Reese et al. (2018) failed to detect a subspecies effect when using cows with $\frac{3}{4}$ to $\frac{1}{8}$ *Bos indicus* influence. Differences between maternal PAG circulating concentrations can also be found by breed where Hereford cows tended to have greater levels during the peripartum period when compared to Holstein cows (Zoli et al., 1992). Additionally, changes in maternal PAG circulating concentrations have been detected within subsequent pregnancies of high fertility heifers when subjected to FTET, suggesting that PAG concentrations are not repeatable across pregnancies (Reese et al., 2018).

2.2. Enzyme-linked immunosorbent assay

Each unique PAG is considered a placental antigen that has the capacity bind to an antigen-specific antibody and allow for its detection (Sasser et al., 1986; Sasser,

Crock, and Ruder-Montgomery, 1989). This allows for the application of an immunoassay known as enzyme-linked immunosorbent assay (ELISA) as a pregnancy test (Green et al., 2005). Moreover, the usage of immunolocalization processes to identify PAG expression and accumulation patterns (Wooding, Roberts, and Green, 2005). Given the vast family of genes present in the PAG family, it is not a surprise that PAGs in the bovine and ovine species are known for demonstrating different spatial and temporal expression patterns throughout gestation (Green et al., 2000; Telugu, Walker, and Green, 2009; Touzard et al., 2013). For instance, although PAG-1 was the first one discovered (Butler et al., 1982) it does not imply it being the first PAG expressed by trophoblast cells during pregnancy in ruminants, nor that bovine PAG-10 and caprine PAG-10 have any ortholog origin (Wallace, Pohler, Smith, and Green, 2015). Bovine PAG-1 was predominantly used for pregnancy tests; however, given that bovine PAG-1 may not be secreted during early stages of gestation, further research suggested that the antiserum used for these tests may detect other types of PAGs (Green et al., 2000). Zoli et al. (1992) demonstrated that bovine PAG-1 maternal circulating concentrations reached undetectable levels until days 80-100 postpartum. Moreover, studies identify bovine PAG-1 half-life between 7.3 and 8.4 days after parturition (Sasser et al., 1986; Kiracofe et al., 1993) and 7 days after induction of embryonic mortality (Semambo, Eckersall, Sasser, and Ayliffe, 1992). Combining the increased quantity of PAGs found at time of parturition with its long half-life, false positives can be a noticeable disadvantage, leading to a clear disruption in using PAG-1 as an early pregnancy diagnosis method.

Green et al. (2005) established an in-house sandwich ELISA to detect early secreted PAGs with shorter half-lives. Sandwich ELISAs are able to measure the amount of antigens (PAGs) that are located between two antibodies (Vanmechelen et al., 2000; Silva et al., 2009). For this assay, PAGs are trapped in between a set of previously purified monoclonal antibodies (A6, J2, L4) and a detecting polyclonal antibody raised against mid-gestation secreted PAG genes. Pregnancy associated glycoproteins bound to a polyclonal antibody are detected by using an alkaline phosphatase conjugated anti-rabbit antibody. From the purified monoclonal antibodies, A6 demonstrated a wider affinity against PAGs found in placental tissue, as opposed to J2 and L4 antibodies. Early secreted PAGs detected by A6 monoclonal antibody were PAG-7, -6, -16, -4, -17, -20, and PAG-21. Similarly, PAG-20 was bound to J2. Pregnancy associated glycoproteins detected by L4 monoclonal antibody were PAG-16, -17, and PAG-2. Reactivity of PAGs against the monoclonal antibodies were specifically to those pertaining the "modern" group that are primarily BNCs expressed. Additionally, by week 8 after parturition its circulating concentrations reached levels below the established threshold used to confidently determined pregnancy. The half-life of these PAGs averaged 4.3 days after parturition, lowering the possibilities of a false positive on days 28 of gestation. Studies from Pohler et al. (2013) and Szenci et al. (2003) found different half-lives for bovine PAG after induction of pregnancy loss; 1.5 days and between 2.7 to 3.9 respectively.

2.3. Antibody dependence

Green et al. (2005) results using different purified monoclonal antibodies, as stated previously, gave rise to an antibody dependence idea to detect different and more accurate PAG genes. A subsequent study by Pohler et al. (2013), concluded not only a dependency on a monoclonal antibody, but also in the polyclonal antibody used to detect PAGs. This experiment followed the same sandwich ELISA protocol; however, using a different polyclonal detecting antibody, known as antibody (Ab) 45, than the one described in Green et al. (2005). Giving the possibility that the assay detected different types of PAGs; it was suggested that they were more early secreted ones as per its shorter half-life (1.5 days) compared to Green´s 4.3 days. To validate the results indicating an antibody dependence in the detection of PAGs, Gatea et al. (2018) used different combinations of antibodies in an in-house sandwich ELISA as well as a commercial kit on FTAI and FTET groups. The monoclonal antibodies used to trap PAGs were A6, J2 and L4; same as previous experiments. Specific differences in antibodies were on a polyclonal detecting antibody level, using Pohler et al. (2013) Ab 45 and a new Ab F2. The combinations used were as follows: Mix-45 (A6, J2, L4 monoclonal antibodies with 45 polyclonal antibody), Mix-F2 (same set of monoclonal antibodies with an F2 polyclonal antibody), L4-F2, and a commercial kit (IDEXX). Each polyclonal detecting antibody were created using specific and unique antigens, main reason behind each antibody´s specificity in detecting PAGs. For instance, polyclonal antibody 45 and F2 consist of a mixture between antigens raised against PAG 4, 6, 7, 16 and 20, additionally ab F2 can detect PAG2. The IDEXX commercial kit has a

combination of PAG 4, 6, 9, 16, 18, and 19 (US patent no. 7,604,950B2). Results from the FTAI group showed that only the mixture using the polyclonal detecting Ab 45 was able to predict embryonic mortality between days 30-60 of gestation. Whereas the FTET group experienced somewhat similar results when using Ab 45; however, the IDEXX commercial kit was also able to predict embryonic mortality. Both assays had a 95% accuracy although maternal circulating PAG concentrations for pregnancy diagnosis as well as concentrations to predict late embryonic mortality were decreased in the IDEXX commercial kit: 1.19 ng/mL and >4.72 ng/mL respectively. Variations in results demonstrate the affinity of antibodies in detecting different PAGs in early stages of gestation and how each unique PAG may or may not be associated to embryonic mortality. Although, it is not a dismissing factor of the accuracy that each combination of antibodies or the commercial kit has in detecting pregnant vs non pregnant animals.

A further study compared different polyclonal detecting antibodies efficiency on identifying circulating PAGs on day 24 of gestation, as opposed to Gatea et al. (2018) which used samples from day 30. Reese et al. (2018) applied the same sandwiched ELISA protocol established by Green et al. (2005) and later modified by Pohler et al. (2013), previously specified as Ab 45, with an addition of a new polyclonal antibody raised against early secreted PAGs known as Ab 63. Both antibodies were able to efficiently detect pregnancy diagnosis at a 90% and 95% accuracy; however, Ab 45 had decreased thresholds in which animals were considered pregnant. Resulting in a more precise diagnosis by utilizing Ab 45; differences attributed to the range of PAGs detected by each specific antibody (Gatea et al., 2018). Fontes et al. (2019) also found

differences in maternal circulating PAGs when using two commercials kits (BioPRYN and IDEXX) and the in-house ELISA using Ab 63. BioPRYN commercial kit mainly detects PAG1 while the IDEXX kit has a wider range of detection as previously stated. Embryos from Brangus and Angus cows were used to compare its effects on circulating PAGs from day 28 through 91 of gestation. When utilizing BioPRYN commercial kit, Brangus embryos had greater PAG concentrations than Angus embryos only on day 91 of gestation; however, when using the IDEXX kit, Brangus embryos had greater concentrations throughout the whole experiment. On the other hand, the in-house sandwich ELISA with Ab 63, Angus embryos had greater PAG concentrations on day 25 and 28 of gestation only. Differences were also detected on false positives cases in pregnancy diagnosis on day 28 of gestation, with Reese et al. (2018) having 55% when using an in-house sandwiched ELISA compared to the 6% reported by Middleton and Pursley (2019) with a BioPRYN commercial kit. Reaffirming the theory that PAG results are highly dependent by the antibody used in the assay and that PAG concentrations are influenced by breed.

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3. EFFECT OF EMBRYO SIZE ON BOVINE PREGNANCY ASSOCIATED GLYCOPROTEINS THROUGHOUT DIFFERENT STAGES OF GESTATION

3.1. Synopsis

Pregnancy associated glycoproteins (PAG) are trophoblastic proteins used as biomarkers for pregnancy diagnosis and predicting embryonic mortality in cattle. The exact function of these glycoproteins remain unclear, but it is suggested to be a marker for placental function. Therefore, the objective of this experiment was to determine the relationship between circulating PAG concentrations and embryonic-fetal size and development throughout different stages of gestation. Blood samples and pregnancy diagnosis and status was performed via transrectal ultrasound on a total of 137 multiparous beef cows at three different locations. Studied days varied according to the location with Location 1 being days 28 and 67, Location 2 being days 35 and 53, and Location 3 being days 35, 42, 49, 56, 63, 70, 77, 84, and 91 of gestation. Embryo crown to rump length (CRL) was measured for each of the selected days and PAG concentrations were analyzed using an in-house sandwich ELISA. Data was analyzed using PROC CORR (SAS 9.4). Embryonic-fetal CRL was not significantly correlated to PAG concentrations at any of the three locations, except for PAG on day 42 with CRL day 70 on Location 3 (r=-0.41; *P*=0.01). Day 28 and 67 PAG concentrations were not associated with birthweights at Location 1 (r=-0.24; *P*=0.12 and r=-0.11; *P*=0.47, respectively). Embryonic growth at Locations 1 and 2 was not associated with PAG concentrations. A relationship between embryo-fetal growth was detected at Location 3

between PAG on day 35 and growth between days 35 and 42 (r=0.35; *P*=0.04), and PAG on day 42 and growth between days 63 and 70, and 70 and 77 (r=0.37; *P*=0.03 and r=-0.41; *P*=0.01, respectively). The difference in PAG concentrations between days 35 and 42 was correlated with embryo CRL on day 35 of gestation (r=0.53; *P*=0.001). According to these data, PAG concentrations are not associated with embryo-fetal size or development throughout gestation.

3.2. Introduction

In ruminants, pregnancy associated glycoproteins (PAG) are proteins secreted by trophoblast giant cells into the maternal circulation (Wooding, 1981; Wooding, 1983; Zoli, Ectors, and Beckers, 1990). The identification of the first PAG, bovine PAG1 or otherwise known as Pregnancy-specific protein B (PSPB), offered a base line to a series of attempts trying to identify a useful purpose for such markers (Butler et al., 1982). Currently, assays that detect circulating concentrations of PAGs in blood or milk samples, have an accuracy of 98-99% when detecting pregnant versus non-pregnant cattle between days 28 and 30 of gestation (Green et al., 2005; Pohler et al., 2013; Wallace, Pohler, Smith, and Green, 2015). Moreover, pregnancy diagnosis using PAG concentrations as early as day 24 of gestation is an option; however, this early diagnosis can overlook incidences of early embryonic mortality, thus generating misleading results (Arnold et al., 2012; Reese et al., 2018; Oliveira Filho et al., 2020).

Given the economic loss associated with embryonic, several studies identified an association between accurate prediction of embryonic morality with PAG concentrations at day 28 and 30 of gestation (Szenci et al., 2003; Pohler et al., 2016b). In dairy cattle,

Breukelman et al. (2012) identified that reduced concentrations of circulating PAGs between days 25 and 32 of gestation was associated with animals that experienced pregnancy loss between days 26 and 120 of gestation; however, a threshold was not determined. In beef cattle, Pohler et al. (2013) detected an increased likelihood of pregnancy maintenance for cows with greater circulating PAG concentrations on day 28 of gestation. Additionally, Pohler et al. (2016b) and Pohler et al. (2016a) determined a threshold level of circulating PAGs around day 30 of gestation in dairy and beef cattle with an accuracy of 95% in predicting late embryonic mortality when compared to transrectal ultrasound pregnancy diagnosis. Similarly, Gatea et al. (2018) was 95% accurate in predicting late embryonic mortality using PAGs on day 30 of gestation in dairy cattle after both fixed-time artificial insemination (FTAI) and fixed-time embryo transfer (FTET). Nevertheless, the threshold identified for predicting embryonic mortality for FTAI was greater than that of Pohler et al. (2016a). Even though several studies have linked embryonic loss or pregnancy maintenance to maternal circulating PAG concentrations, the causal effect explaining this relationship is yet to be determined.

The physiological role(s) of PAGs are not fully understood, with some suggesting an influence on the maternal immune system, protection of the corpus luteum, control of growth factors involved in the placental-uterine interface, and influence on placental function and/or efficiency (Austin et al., 1999; Hoeben et al., 1999; Chavatte-Palmer et al., 2006; Constant et al., 2011; Pohler et al., 2013; Wallace, Pohler, Smith, and Green, 2015). Placental attachment in bovine species occurs around

day 17 after fertilization (Roberts, Xie, Mathialagan, 1996) and factors negatively affecting this process can create a disruption in the uterine-placental interface and thus affect proper maternal-fetal exchanges (Vonnahme, 2007). This disruption can adversely alter fetal, neonatal and/or postnatal development (Summers and Funston, 2013; Du et al., 2015). In some cases, embryo development from cloned-derive or *in vitro* produced (IVP) embryos is increased late in gestation compared to normal embryos and has been linked to Large Offspring Syndrome (LOS) (Heyman et al., 2002; Lazzari et al., 2002; Lee et al., 2004). Moreover, these types of embryos show variations in their PAG concentrations (Chavatte-Palmer et al., 2006; Constant et al., 2011; Pohler et al., 2016a; Gatea et al., 2018).

Although there is limited evidence that greater circulating PAG concentrations is associated with improved placental function, LOS is associated with placental dysfunction and affects PAG concentrations which may suggest an association between placental function, embryo size and maternal circulating PAG concentrations. However, a preliminary study by Pohler et al. (2014) rejected this idea by finding no relationship between embryo size and serum PAG concentrations on day 35 and 56 of gestation. Based on the literature available, the objective of this experiment was to determine the correlation between embryonic and fetal size and circulating PAG concentrations on different days of gestation. Our hypothesis was that the size of embryo and fetuses does influence production of PAG.

3.3. Materials and methods

3.3.1. Locations

3.3.1.1. Location 1

The experiment was conducted in 2019 at Texas A&M University with all procedures following Texas A&M University Institutional Animal Care and Use Committee guidelines and animals were kept at Texas A&M University- Beef Cattle System. A total of 196 multiparous beef cows were submitted to a 7 d CO-Synch + CIDR® synchronization protocol for FTAI (Lamb et al., 2006). Day of FTAI was considered as gestation day 0. Pregnancy diagnosis by detection of embryonic heartbeat was assessed on day 28 of gestation. Any open cows were removed from the experiment. Pregnancy status was confirmed on day 67 of gestation, cows that experienced late embryonic mortality (LEM) were removed from the experiment. A total of 67 cows were eligible by the end of the experiment.

3.3.1.2. Location 2

The experiment was conducted in 2016 at the University of Tennessee, all procedures followed the recommendations of the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 2010) and animals were kept at Middle Tennessee Research and Education Center. A total of 115 beef multiparous cows were submitted to a 7 d CO-Synch + CIDR® synchronization protocol for FTAI (Lamb et al., 2006) . Pregnancy diagnosis was obtained on day 35 of gestation and subsequently verified on day 53. A total of 67 cows were eligible by the end of the

experiment after removing any open cows on day 35 and cows that experienced embryonic loss on day 53 of gestation.

3.3.1.3. Location 3

The experiment was conducted over the course of two years (2015 and 2016) at the University of Florida, all procedures were approved by the university of Florida Institutional Animal Care and Use Committee and animals were kept at University of Florida – Feed Efficiency Facility at the North Florida Research and Education Center. A total of 197 recipient beef cows were submitted to the 7 d CO-Synch + CIDR® synchronization protocol (Lamb et al., 2006). A presence of a corpus luteum was verified on day 7 after initiation of the protocol and fresh transfer *in vivo* embryos were transferred to eligible cows (n=96, year 2015 and 101 in 2016). Pregnancy diagnosis by detection of embryonic heartbeat was assessed on day 28 of gestation. Further pregnancy status was confirmed on days 35, 42, 49, 56, 63, 70, 77, 84, and 91 of gestation. After removing open cows on pregnancy diagnosis day, cows that experienced embryonic mortality on subsequent pregnancy status, and animals where data was missing, only 35 cows were eligible for the experiment.

3.3.2. Blood sampling and pregnancy associated glycoproteins

Blood samples from Location 1 and Location 2 were collected on each specific pregnancy diagnosis and pregnancy status day by tail venipuncture using a 10 mL vacutainer tube (BD Vacutainer® Serum, Becton, Dickinson and Company, NJ) for serum samples. Blood samples from Location 3 were collected from the jugular vein using 10 mL vacutainer tubes containing 143 IU of sodium heparin (Vacutainer; Becton,

Dickinson and Company, Franklin Lakes, NJ) for plasma samples. Samples were centrifuged at 2,000 x *g* for 15 minutes at 4 ºC. Serum samples were transferred to microtubes while plasma samples into polypropylene tubes at -20 ºC for each cow. Pregnancy associated glycoprotein concentrations were quantified using an in-house ELISA assay established by Green et al. (2005) with a different polyclonal antibody, Ab 63, as used by Reese et al. (2018) and run in duplicates. A standard curve was used for each plate along with positive (pooled sample from a pregnant cow with more than 60 days of gestation) and negative (pooled sample from a steer or a non-pregnant cow) controls. An ELx808 microplate reader (Bio-Tek, Winooski, VT) was used to read the absorbance from each samples at 405 nm.

3.3.3. Pregnancy diagnosis and embryo morphometry

All pregnancy diagnosis was assessed by utilizing an IBEX EVO® transrectal ultrasound with a linear 5 probe MHz probe. On the same day of pregnancy diagnosis and pregnancy status, videos were taken of the embryo-fetus and saved. Crown to rump length (CRL) and embryo area images from each day of gestation were measured using the IBEX EVO® ultrasound. Images of fetal heads from days were taken when the full length of the embryo could no longer be assessed and were measured using the IBEX EVO® ultrasound. Head diameters were transformed to CRL measurements using Riding, Lehnert, French, and Hill (2008) formula: $y= 0.3203x + 3.3978$. With x = CRL (mm). Location 2 measurements were performed by a different technician than location 1 and 3.

3.3.4. Statistical analysis

An analysis of correlation using PROC CORR of SAS (SAS 9.4. Institute Inc., Cary, NC) was used to detect associations between factors. Results will be presented as a Pearson correlation coefficient (*r value*). Significant correlations will have a $P < 0.05$. Tendencies will be considered at $0.05 \leq P \leq 0.10$.

Crown to rump lengths diameters on Location 1 from days 28 and 67 were correlated with respective PAG (ng/mL) concentrations. The same analysis was used to compared PAG (ng/mL) concentration on day 28 of gestation with differences in growth between day 28 and 67 of gestation and with birthweight. Location 2 diameters from CRL were correlated with respective PAG (ng/mL) concentrations on days 35 and 53. The same analysis was used to compared PAG (ng/mL) concentration on day 35 of gestation with differences in growth between day 35 and 53 of gestation. Crown to rump length diameters from Location 3 on days 35, 42, 49, 56, 63, 70, 77, 84, and 91 were correlated with respective PAG (ng/mL) concentrations. Same analysis was used to compared PAG (ng/mL) concentration on day 35 and 42 of gestation with differences in growth between subsequent days of gestation (e.g. growth between days 35-42, 42-49, 49-56, 56-63, 63-70, 70-77, 77-84, 84-91, 35-91, and 42-91). Likewise, a correlation was calculated between fetal measurements on days 35, 42, 49, 56, 63, 70, 77, 84, and 91 with the difference in PAG concentrations between days 35 and 42 of gestation.

3.4. Results

3.4.1. Location 1

Summary of Location 1 results can be found in Table A1. Mean maternal circulating PAG concentrations at day 28 of gestation was 7.92 ± 3.84 ng/mL, while day 67 had a mean concentration of 6.09 ± 3.75 ng/mL. There was not a significant correlation ($P = 0.58$) between day 28 serum PAG concentrations and embryo sizes, the same observations were found for day 67 of gestation ($P = 0.89$). A relationship among PAG concentrations on day 28 of pregnancy with embryonic growth between days 28 and 67 was not detected ($P = 0.48$). Likewise, PAG concentrations on day 28 and 67 of gestation was not associated with birthweight ($P = 0.12$).

3.4.2. Location 2

Summary of Location 2 results can be found in Table A2. Maternal circulating PAG concentrations on day 35 of gestation did not have a significant correlation with embryo size ($P = 0.43$), nor did it have an effect on embryonic development from day 35 until day 53 of gestation ($P = 0.36$). Fetal CRLs from day 53 of pregnancy were not associated with maternal circulating PAG concentrations on the same day ($P = 0.24$). Although an effect was not present in any of the correlations, a considerable decrease was observed on mean serum PAG concentrations between days 35 and 53; from 8.81 to 2.77 ng/mL respectively (Figure B2).

3.4.3. Location 3

Mean plasma PAG concentrations decreased from day 38 until day 49 of gestation, slight increases were detected afterwards (Figure B3). A significant

correlation was not detected between PAG concentrations on days 35, 42, 49, 56, 63, 70, 77, 84, and 91 with their respective CRLs (Table A3). The same results were found when comparing PAG concentrations on day 35 with fetal development on subsequent days, except for the growth between day 35 and 42 of gestation were a significance was detected ($P = 0.03$; Table A4). Moreover, a tendency was identified between PAG concentrations on day 35 of gestation with embryonic growth between days 70 and 77 ($P = 0.08$; Table A4). A similar outcome was observed when correlating maternal circulating PAG concentrations on day 42 with CRL on day 70 (P = 0.01; Table A3) and the embryonic-fetal growth between days 63 to 70 ($P = 0.02$; Table A4) and days 70 through 77 (P = 0.03; Table A4) of gestation; however, significance correlations were not detected for any other days. Differences in PAG levels between days 35 and 42 of gestation affected embryonic size on day 35 of gestation ($P = 0.001$; Table A5); however, an association was not detected on successive days.

3.5. Discussion

Maternal circulating PAG in blood or milk samples can be successfully used as markers for pregnancy diagnosis in ruminants species, specifically even-toed ungulates (Haugejorden et al., 2006; Sousa, Ayad, Beckers, and Gajewski, 2006; Szafranska, Panasiewicz, and Majewska, 2006). The exact role of these glycoproteins remain unknown; however, it has been suggested as a marker for placental function due to the relationship with embryonic mortality (Pohler et al., 2013). Embryonic mortality or pregnancy loss can be cause by several factors such as heat stress, chromosomal abnormalities, nutrition, infections or hormonal disruptions with most of these factors

impacting maternal-fetal nutrient and gas exchanges (Ayalon, 1981; Hansen, 2002; Bilodeau-Goeseels and Kastelic, 2003). The maternal-fetal nutrient and gas exchange is achieved by the placenta, a pregnancy-developed organ by which any disruption of its tissue can affect fetal growth (Bell and Ehrhardt, 2002; Wu et al., 2004; Che et al., 2017). Pregnancy associated glycoproteins may have an association with embryo size considering that PAG concentrations are related with placental function and uterine environment which affects fetal growth. Moreover, several studies have associated lower PAG concentrations with a decrease likelihood of pregnancy loss (Breukelman et al., 2012; Pohler et al., 2013). Raising the question if embryonic mortality related to lower PAG concentrations is indeed driven by a smaller embryo.

Pohler et al. (2014) failed to detect a relationship between PAG concentrations and embryo size on days 35 and 53 of gestation. In the present study, similar results were obtained for the three locations. Pregnancy associated glycoproteins secretions start between days 19 and 21 of gestation and increases rapidly from day 24 until approximately day 36 when levels begin to decrease (Wooding, 1983; Pohler et al., 2013; Seo, Bazer, Burghardt, and Johnson, 2019). Similar observations were made at Locations 2 and 3. Concentrations begin increasing again mid-gestation until term; however, the exact mechanism remains unclear. Pregnancy associated glycoproteins concentrations have been detected in virgin heifers and bulls, suggesting the presence of PAGs in foreign tissues that are triggered and release in pregnant cows closer to parturition (Sasser et al., 1986; Zoli et al., 1992; Green et al., 2005). Additionally, it

could be due to an increase in placental size (Prior and Laster, 1979; Green et al., 2005; Pohler et al., 2013).

Contrary to this, fetal development follows a sigmoid curve but with exponential growth until its size stabilizes closer to parturition (Richardson, Barnard, Jones, Hebert, 1991; Gogan et al., 2005). Discrepancies in results between day 35 and before mid-gestation may be due to the inverse relationship between PAG concentrations and embryo size. Evaluation before day 35 of gestation was only done at Location 1 where day 28 embryo size was compared to PAG levels; however, no significant relationship was detected. Further experiments should analyze PAG concentrations and embryonic size at selected stages of increasing PAG levels in gestation; however, disadvantages may be present by the existing in-house sandwich ELISAs which detect early secreted PAGs that may not be present later in gestation.

In general, placental function as well as any other nutrient related systems, do not convey an instant response into its target tissue. Several studies have associated an increase in maternal circulating PAG concentrations around day 28 and 30 of gestation to a decreased probability of embryonic loss later in gestation (Thompson et al., 2010; Breukelman et al., 2012; Pohler et al., 2014). Moreover, placental abnormalities formed during placental development (e.g. disrupted angiogenesis, lower placentome numbers and vascularity) associated with IVP and cloned-derived embryos are involved in pregnancy loss later in gestation (Heyman et al., 2002; Farin, Piedrahita, and Farin, 2006). For these reasons, looking at PAG concentrations correlated to the respective embryonic size on the same day of gestation may overlook fetal growth associated to

placental efficiency. In the present study, Locations 1 and 2 did not have a significant correlation between serum PAG levels and embryonic sizes on different days of gestation. The days of gestation used for these studies may have influence the results. As it was previously stated, PAG concentrations do not follow an increasing linear type of secretion as opposed to the sigmoid pattern of fetal growth.

Location 3 had similar results when utilizing PAG concentrations on day 35 against crown to rump lengths later in gestation; however, PAG concentrations on day 42 did have a significant correlation with embryonic size on day 70 of gestation. The association with PAG concentrations on day 42 with fetal size on day 70 may be explained by the time and capacity of placental remodeling. A correct bovine placental remodeling is necessary to maintain proper nutrient and gas exchanges that will affect fetal development (Burton, Fowden, and Thornbug, 2016). In ruminants these exchanges happen in the placentomes; with abnormal morphologies and lower counts associated with placental deficiencies (Edwards et al., 2003; Miles et al., 2004). The number of placentomes in the bovine placenta are around 20 between days 37 and 40 of gestation, with a substantial increase in numbers from day 40 to 50 of gestation (Farin and Farin, 1995; Asiss Neto, Santos, Pereira, and Miglino, 2009; Peter, 2013).

A more recent study found the same trifold increase in placentome numbers from day 48 until day 157 of gestation; associated with the rapidly increasing fetal growth (Estrella et al., 2017). Pregnancy associated glycoprotein concentrations on day 42 fall between the days of rapid placentome increase associated with placental efficiency and embryonic-fetal growth later in gestation, explaining the significant correlation between

PAG concentrations on day 42 and fetal size on day 70 of gestation. Pregnancy associated glycoproteins concentrations on day 42 of gestation also affected fetal growth between days 63 to 70 and days 70 to 77. A positive correlation was detected between days 63 and 70 while days 70 to 77 had a negative association attributed to a lack of statistical power.

As previously stated, PAGs are secreted a few days after implantation and have a rapid increase from day 24 until day 36 (Wooding, 1983; Pohler et al., 2013; Seo et al., 2019). Bovine implantation occurs from day 19 to day 21 post-fertilization, during this stage and on following days the embryo will undergo organogenesis and increase in size (Senger, 2012; Valadão, da Silva, and da Silva, 2018). Bovine embryos will grow from around 0.196 mm at day 8 post-fertilization to approximately 13.5 to 15 mm on day 35 post-fertilization (Hamilton and Laing, 1946; Wrobel and Süß, 1998; Assis Neto et al., 2010). This data goes in accordance with day 35 embryonic measurements from Locations 2 and 3. Increases in sizes and nutritional requirements must be accompanied by correct placental efficiency, which has been related to PAG concentrations, suggesting a relationship between PAG levels and embryonic growth during early stages.

Location 3 detected an early effect between both factors when comparing PAG concentrations on day 35 of gestation against the embryonic growth between days 35 and 42 of gestation and a tendency between days 70 and 77 of gestation. A significant association was detected when the difference between PAG concentration was correlated with crown to rump length of day 35 embryos. Although significance in the previously

mentioned instances, they were only detected in Location 3; however, the strength of the correlations was not powerful enough to conclude a definite relationship between both factors. Nonetheless, significant associations found only on Location 3 compared to Location 1 and 2 may also be due to the differences in PAG concentrations between blood sample types; with plasma samples having more than three times the concentrations found in serum (Wesson et al., 2020). Further studies must be done in order to determine a more robust correlation between maternal circulating PAG concentrations and embryonic-fetal growth throughout different days of gestation.

Placental deficiencies can disrupt nutritional supplement to the fetus, thus development and subsequent birthweight (Ferrel, 1993; Bertolini and Anderson, 2002). Pregnancy associated glycoproteins have been proposed as markers for placental function; with the theory that increased levels of PAGs in the circulation is related to an increase in placental efficiency. The present study attempted to detect a relationship between PAG levels and birthweights on Location 1; however, it failed to detect one.

3.6. Conclusion

In conclusion, maternal circulating PAG concentrations are not driven by embryonic-fetal size measurements throughout different stages of gestation. For this reason, embryonic loss associated to lower maternal circulating PAG concentrations does not seem to be caused by a smaller embryo. Moreover, the usage of PAG concentrations to infer gestational age is not a plausible option. Although a correlation was detected on certain days when compared to a specific PAG concentration, the strength of the correlation was not powerful enough to conclude an existing relationship

between both factors. A better approach for further studies would be to analyze PAG concentrations with embryo size and development on days of gestation where both factors experience a direct increase.

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4. EFFECT OF VARIOUS METHODS OF HANDLING OF PLASMA AND SERUM SAMPLES ON BOVINE PREGNANCY ASSOCIATED GLYCOPROTEINS

4.1. Synopsis

Early pregnancy diagnosis and embryonic loss in cattle can be determined by using pregnancy associated glycoproteins (PAG). Therefore, it is critical to analyze factors that may influence PAG concentrations results and diagnosis. The objective was to determine any variations in PAG concentrations between serum and plasma samples when being subjected to different centrifugation days, repeated freeze-thaw cycles, and using monoclonal antibodies that were kept in frequently or infrequently opened containers. Blood samples were collected from a total of 4 multiparous cows, two pregnant and two open cows, after pregnancy diagnosis was performed on day 28 of gestation via transrectal ultrasound. Different collecting tubes were used to obtain either plasma or serum samples and evenly distributed to be centrifuged on the same day of collection or incubated for 24 hours in a 4ºC refrigerator before centrifugation. After centrifugation and before freezing, 1 mL from all samples was taken and frozen at -20ºC (INTACT). The remaining sample (NOTHAW) was analyzed using an in-house sandwich ELISA and subsequently frozen. The process was repeated after one freeze-thaw cycle (THAW1) and two freeze-thaw cycles (THAW2). One week after, samples had a third freeze-thaw cycle (THAW3) and analyzed with the INTACT sample. A one-way ANOVA PROC GLM, a LSMEANS, and a Tukey Test (SAS 9.4) were used to analyze the data. All open cows were below the baseline of the assay and excluded from

the results. Plasma PAG concentrations were higher than serum concentrations ($P<0.0001$). Multiple freeze-thaw cycles did not affect overall plasma concentrations ($P=0.19$) while serum concentrations were affected ($P=0.03$). Serum THAW1 and THAW2 differed from the NOTHAW sample ($P=0.02$ and $P=0.01$, respectively) while THAW3 was not different from the INTACT or NOTHAW sample ($P=0.21$ and $P=0.85$, respectively). Centrifugation day did not affect plasma or serum samples ($P=0.50$ and $P=0.59$, respectively). Frequency of usage between monoclonal antibodies did not influence PAG concentrations ($P=0.89$). In summary, plasma PAG concentrations are more stable than serum when subjected to repeated freeze-thaw cycles. Moreover, day of centrifugation and handling of monoclonal antibodies does not altered PAG concentrations in either plasma or serum samples.

4.2. Introduction

Pregnancy associated glycoproteins (PAGs) are trophoblastic proteins released from the placenta into the maternal circulation in ruminant species (Wooding, 1981; Zoli et al., 1992). Pregnancy in cattle can be currently diagnosed by measuring circulating PAGs in blood or milk samples between days 28 and 30 of gestation with a 98-99% accuracy (Green et al., 2005; Pohler et al., 2013; Wallace, Pohler, Smith, and Green, 2015). Moreover, numerous studies have detected a relationship between late embryonic mortality and PAG concentrations on day 28 and 30 of gestation; with decreased levels of PAGs being associated with an increased risk of pregnancy loss (Thompson et al., 2010; Breukelman et al., 2012; Pohler et al., 2013; Pohler et al., 2014; Pohler et al., 2016a; Gatea et al., 2018; Reese et al., 2019). However, a relationship between PAG

concentrations and embryonic mortality was not detected by Ricci et al. (2015). Given the contradicting results between studies, research has looked to identify possible causes of these differences.

Blood samples used when analyzing PAGs are centrifuged and either plasma or serum is extracted for the assay. Utilization of either plasma or serum does not appear to alter pregnancy diagnosis around day 30 of gestation in cattle (Thompson et al., 2010; Pohler et al., 2013; Ricci et al., 2015; Commun et al., 2016) or sheep (Steckeler et al., 2019). As previously stated, serum PAG concentrations is correlated with pregnancy loss (Pohler et al., 2013; Pohler et al., 2016b); however, Ricci et al. (2015) failed to detect a relationship when using plasma samples. This suggests that levels of PAG between serum and plasma samples may affect outcomes related to predicting late embryonic mortality. It is proven that most protein concentrations are greater in plasma samples when compared to serum (Andreasen, Latimer, Kircher, and Brown, 1989; Kaneko, Harvey, and Bruss, 2008); however, an actual difference in serum or plasma PAG concentrations has yet to be established.

In addition to potential differences between plasma and serum, freeze-thaw cycles of samples can also influence results. In a laboratory setting, blood samples will sometimes be processed for more than one type of assay that cannot be performed on the same day, thus leading to several freeze-thaw cycles. Mitchell et al. (2005) detected a change in plasma proteome integrity when subjected to freezing and thawing processes in a short interval of time; however, long term storage had minimal to no effect on total protein. Serum cytokines, which are small proteins, are also affected by this

phenomenon leading to an increase in cytokine levels (Lugos, Polit, and Damen, 2018). It has been demonstrated that repeated freeze-thaw cycles can alter protein stability in serum and plasma samples by denaturation, degradation or functional inactivation (Kisand et al., 2011; Lee, Kim, and Shin, 2015).

Given the importance of accurate detection of PAG levels for pregnancy diagnosis and as a marker for embryonic loss, the primary objective was to determine whether there are any differences in PAG concentrations between serum and plasma samples when being subjected to different centrifugation days and repeated freeze and thaw cycles post-collection. A secondary objective was to determine the influence of monoclonal antibodies that were kept in frequently or infrequently opened containers. Our hypotheses were that 1) centrifugation day and/or freeze/thaw cycles will alter PAG concentrations in serum and plasma samples and 2) that frequency of antibody usage, when contamination is avoided, does not affect in-house ELISA PAG concentrations.

4.3. Materials and methods

4.3.1. Experiment design

All animals were located at Texas A&M university Beef Center and kept in pastures with ad libitum access to water. Pregnancy diagnosis was stablished in animals by detecting an embryonic heartbeat using a transrectal ultrasound (IBEX EVO ®) on day 28 of gestation after being submitted to a commercial FTAI protocol. A total of 4 multiparous cows were used, 2 non-pregnant cows and 2 pregnant cows.

4.3.2. Blood sampling and centrifugation effect

Blood samples were collected from the jugular vein into four 20-mL Vacutainer tubes (BD Vacutainer® Serum, Becton, Dickinson and Company, NJ) to collect serum samples and six 6 mL Vacutainer tubes (BD Vacutainer® K2 EDTA, Becton, Dickinson and Company, NJ) to collect plasma samples. The procedure was repeated for each pregnant and non-pregnant cow. To detect an influence by centrifugation day, centrifugation was held at 2,000 x *g* for 15 minutes at 4 ºC and serum or plasma samples stored in 5 mL microtubes in a -20 ºC freezer; with 2 serum tubes centrifuged on the same day and two serum tubes stored at a 4 ºC refrigerator for 24 hours and centrifuged on the next day. Plasma tubes were subjected to the same procedure

4.3.3. Thawing and freezing effect

To detect an influence by repeated thawing and freezing cycles, serum and plasma samples were firstly analyzed for PAG concentrations before freezing (NOTHAW). On the following day samples were considered as having one thawing process (THAW1) and analyzed again for PAG concentrations and freeze after usage. A day after the first thawing, samples were considered to have a second thawing process (THAW2) and analyzed for PAG concentrations and subsequently frozen. A week following the last freezing day, samples were thawed for a third time (THAW3) and analyzed for PAG concentrations and freeze right after. Additionally, a sample was taken right after centrifugation, before a PAG analysis, and kept frozen in microtubes in a -20ºC freezer. This sample was considered as INTACT since it was not subjected to

any freeze/thaw cycles and subsequently analyzed with THAW3 samples. Similar procedures were applied to samples centrifuged 24 hours after collection day

4.3.4. Monoclonal antibody effect

To detect an influence by frequently used monoclonal antibodies (FUMA) and infrequently used monoclonal antibodies (UUMA), a 96 well plate (Thermo Fisher Scientific, Rochester, NY) was subjected to both antibodies in an in-house sandwiched ELISA assay. Both sets of monoclonal antibodies (A6, J2, L4) are identical in composition; however, FUMA have been constantly used for experimental analysis. On the other hand, UUMA have been safely stored in a 4 ºC refrigerator without being used for any analysis. Forty-eight wells were used with FUMA while the other 48 wells used UUMA. This procedure was only used on the NOTHAW samples.

4.3.5. Pregnancy associated glycoprotein assay

Pregnancy associated glycoprotein concentrations were quantified using an in-house sandwiched ELISA assay established by Green et al. (2005) and modified by Reese et al. (2018) using a different polyclonal antibody (Ab 63) and ran in triplicates. The same standard curve aliquots were used for each plate along with positive (more than 60 days of gestation pregnant cow serum) and negative (steer serum) controls

4.3.6. Statistical analysis

Data was analyzed using a one-way ANOVA, PROC GLM of SAS (SAS 9.4, Institute Inc., Cary, NC) to detect any influence on PAG concentrations between treatments. Treatments were considered as fixed effects and independent variables and PAG concentrations as the dependent variable. Treatments included total concentration

differences in plasma versus serum samples, time of centrifugation between serum and plasma samples, and monoclonal antibody handling. Additionally, a PROC MIXED with repeated measurements of SAS (SAS 9.4, Institute Inc., Cary, NC) was used to detect a difference between INTACT and NOTHAW samples and to detect an influence by repeated freeze/thaw cycles. Significant differences will have a $P < 0.05$. Tendencies will be considered if $0.05 \leq P \leq 0.10$.

4.4. Results

The specific blood type used for samples affected the overall concentration of PAG ($P < 0.0001$; Figure B2), with plasma samples having three times more of the concentration found in serum samples. Plasma had a total of 11.84 ± 2.28 ng/mL (mean ± SD; range 8.21 to 17.06 ng/mL) while serum had 3.296 ± 1.89 ng/mL (mean ± SD; range 0 to 6.68 ng/mL). Apart from having total increased levels, plasma PAG concentrations were not significantly different between the INTACT and NOTHAW sample ($P = 0.41$; Figure B3), nor were they affected by multiple freeze-thaw cycles ($P = 0.28$; Figure B4). Similarly, PAG concentrations from serum samples did not differed between INTACT and NOTHAW samples ($P = 0.25$; Figure B5); however, they changed when exposed to multiple freeze-thaw cycles ($P = 0.01$; Figure B6). A decrease in concentrations was identified on THAW1 and THAW2 compared to NOTHAW ($P = 0.02$ and $P = 0.01$; respectively; Figure B6); however, increased levels were detected on THAW3 that were not different from the NOTHAW samples ($P = 0.86$; Figure B6).

Centrifugation on the same day of collection (CD) vs one day post-collection (CP) did not impact PAG concentrations on plasma samples ($P = 0.50$; Figure B7); with

CD having 11.487 ± 2.37 ng/mL (mean ± SD; range 8.21 to 15.15 ng/mL) and CP 12.194 ± 2.25 ng/mL (mean ± SD; range 9.51 to 17.06 ng/mL). Likewise, PAG concentrations on serum samples were not affected by day of centrifugation ($P = 0.59$; Figure B8). Levels of PAGs from CD had an average of 3.063 ± 1.82 ng/mL (mean ± SD; range 0 to 5.29 ng/mL) while CP concentrations were 3.529 ± 2.03 ng/mL (mean ± SD; range 0 to 6.68 ng/mL).

Pregnancy associated glycoproteins were not affected by utilization of either FUMA or UUMA ($P = 0.89$; Figure B9). Unfrequently used monoclonal antibodies had on average 7.56 ± 7.82 ng/mL (mean ± SD; range 0 to 15.46 ng/mL) compared to 8.17 ± 4.38 ng/mL (mean ± SD; range 4.38 to 12.61 ng/mL) of PAG concentrations obtained when using FUMA.

4.5. Discussion

Whole blood, serum, and plasma are the three sample types that can be used for chemical analysis; however, serum and plasma samples are the preferred choices when performing chemistry testing. Whole blood samples are mainly used with ready-to-go commercial kits for a visual pregnancy diagnosis, although these results can also be used in a laboratory setting to measure PAG concentrations (Mayo et al., 2016). Main differences between both types are the clotting factors and fibrinogen that can only be found in plasma (Siev et al., 2011).

Although circulating PAG levels were increased in plasma samples, pregnancy diagnosis on day 28 of gestation was not influenced by the change in concentrations. Mean serum PAG concentrations were higher than cutoff values used in commercial kits

(1 ng/mL). Results go in accordance with Commun et al. (2016), whom did not detect any influence in pregnancy diagnosis when comparing PAG concentrations between plasma and serum samples on day 30 of gestation. Specificity percentages for both blood types were 100%, while sensitivity was 88.6 % for serum and 88.9% for plasma. Similar findings were determined in small ruminants (i.e. sheep) while performing pregnancy diagnosis mid-gestation; however, specificity between serum and plasma samples was altered with 76.47% and 93.46%, respectively (Steckeler et al., 2019).

Studies have detected an increase in total protein concentrations for plasma samples compared to serum samples in avian species (Andreasen, Latimer, Kircher, and Brown, 1989; Kaneko, Harvey, and Bruss, 2008). It was suggested that higher concentrations of proteins found in plasma may be due to the presence of fibrinogen (Kaneko, Harvey, and Bruss, 2008). Further researchers also found globulin levels having more than three times higher concentrations in plasma samples (Hrubec, Whichard, Larsen, and Pierson, 2002). Moreover, C-reactive protein (CRP) and cytokines like Interleukin 6 (IL-6) and Tumor Necrosis Factor alpha (TNF-α) were increased in plasma compared to serum in humans (Dossus et al., 2009). Plasma PAG concentrations in the present study goes in accordance with these findings; having more than three times the concentrations found in serum.

While pregnancy determination is not affected by different blood types, more sensitive diagnosis like predicting embryonic mortality may be influenced. Pohler et al. (2013) found an increase in likelihood of maintaining pregnancy from day 28 to 72 of gestation in cows that went from 3.14 to 4.53 ng/mL of PAG in serum. That 1.39 ng/mL

increase in PAG levels may be influential in serum samples which contain smaller overall concentrations; however, changes in PAG concentrations in plasma samples may need to be more substantial. Ricci et al. (2015) failed to detect an influence in embryonic mortality by different PAG concentrations when using plasma samples on day 32 of gestation. Even though cutoff values to detect pregnancy by measuring maternal circulating PAG concentrations does not influence the results between plasma or serum samples, a difference between plasma and serum PAG concentrations regarding embryonic mortality should be further studied.

In a comparison study in humans using plasma and serum samples submitted to multiple freeze-thaw cycles (up to five), certain proteins like interferon gamma (IFN-γ) and interleukin-8 (IL-8) were stabled throughout the experiment in both blood type samples (Lee, Kim, and Shin, 2015). The same study found an increasing effect on specific proteins after more than four freeze-thaw cycles in both plasma and serum; however, the increasing rate was more pronounced in plasma samples. Contradictory to these findings, Lugos, Polit, and Damen (2018) found differences in serum concentrations of specific cytokines (including IL-8) after two and three freeze-thaw cycles. Disagreements in IL-8 findings may be attributed to storage and melting temperature variations. The present study did not evaluate freezing, storage or melting temperatures effect on PAG concentrations, and this can be investigated in future experiments. Moreover, these studies suggest that protein concentrations in either serum or plasma samples are specific to the examined protein and can be influenced by temperature. Fluctuations in protein concentrations related to specific proteins have been

furthered analyzed (Kisand et al., 2011). The present study complements these findings by demonstrating that PAG concentrations are not altered by less than four freeze-thaw cycles on plasma; however, serum PAG concentrations were changed. Indicating that protein analysis and its freeze-thaw effect is also specific on the type of blood sample used. Although overall plasma concentrations were not statistically different, there was a difference between PAG concentrations from the intact sample against results from the second freeze-thaw cycle; however, variation was attributed to a small statistical power.

Serum PAG concentrations after the third freeze-thaw cycle increased substantially, similar incidences were found in cytokines concentrations; however, none of them were able to determine an actual reason for the higher incidences (Lee, Kim, and Shin, 2015; Lugos, Polit, and Damen, 2018). Repeated freeze-thaw cycles are known to alter the molecular structure of a protein by breaking its bonds and causing denaturation (Kisand et al., 2011; Lee, Kim, and Shin, 2015). Freezing at different rates forms various sizes of ice crystals, nonetheless, the aqueous solution within the sample will freeze more rapidly causing an increase in solutes concentrations within these "pools" which may change the osmotic pressure and cause protein denaturation (Franks, 1985; Cao, Chen, Cui, and Foster, 2003). During slow rate melting (<10 º C/min) smaller crystals fade prior to larger ones with some of these transforming once again into larger crystals in a process called recrystallization; this sudden restructuration further adds to protein denaturation (Fransen, Salemink, and Crommelin, 1986; Cao, Chen, Cui, and Foster, 2003). Absorption of the protein to the crystal surface provokes a structural change and denaturation; however, this process its reversible to the majority of the proteins after

melting (Chang, Kendrick, and Carpenter, 1996; Strambini and Gabellieri, 1996). Explaining the stable concentrations found throughout the experiment on plasma samples and within the first freeze-thaw cycles of serum.

Although denaturation on proteins caused by repeated freeze-thaw cycles can be reversible (renaturation) up to some extent this process may be altered in serum samples after repeated freeze-thaw cycles. Protein denaturation results in the loss of its main structure into smaller peptides and in most cases loss of biological functionality (Tanford, 1968, 1970). Smaller peptides can still induce an antigen-antibody reaction for the protein of interest (Deaton et al., 2015), as seen in processes were an antibody is raised against synthetic peptides derived from an unavailable protein (Goding, 1996; Lee et al., 2016). Suggesting that the increase in PAG concentrations detected may be caused by denaturation of the protein into smaller peptides, which are increased in numbers, that can still induce an antigen-antibody reaction.

Storing of serum and plasma samples did not affect PAG concentrations when the stored sample was the one not submitted to a previous freeze and thaw cycle. Although storage time was only a weeklong, researches analyzing plasma samples after 4 years of storage had minimal to no effect on total protein concentrations (Mitchell et al., 2005). Even though the experiment did not detect an influence by long-term storing, proteome stability was affected after two freeze-thaw cycles; however, changes were protein specific. Similar results were found in total protein concentrations in serum samples when frozen as an intact sample for up to three months while differences were detected after the first freeze-thaw cycles (Cuhadar, Koseoglu, Atay, and Dirican, 2013).

Suggesting that long-storage of either plasma or serum samples will not affect further analyses if stored as an intact sample or with minimal freeze-thaw cycles. Moreover, an effect on overall results concerning the intensity of the changes is dependent on the desired outcome, as seen how in the present study differences in concentrations did not affect pregnancy diagnosis in cattle.

Apart from differences in fibrinogen and clotting factors, plasma samples can be centrifuged immediately after collection while serum samples need to clot beforehand. In mammals species the National Committee for Clinical Laboratory Standards (NCCLS) recommend a maximum of 2 hours from time of collection until centrifugation on both plasma and serum samples to reduce differences in analyte values (NCCLS, 1990, 1991). These guidelines are factual if a centrifuge is available at the site of collection or relatively closed; however, usually blood and plasma samples will be kept refrigerated for longer intervals of time before processing. In serum and plasma samples, incubation in a 4ºC refrigerator between 1, 12 and 24 hours before centrifugation had an effect on proteome; however, samples from the same incubation intervals had minimal discrepancies between them (Hsieh, Chen, Pan, and Lee, 2006). Differences in analyte values were also detected on serum and plasma samples from avian species incubated for a shorter period of time than the 2 hour standard (Hrubec, Whichard, Larsen, and Pierson, 2002). In the present study, incubation time before centrifugation of either plasma or serum samples at same day of collection or one day post-collection did not affect overall PAG concentrations. Results contradict that of Hsieh, Chen, Pan, and Lee (2006); however, the study only analyzed small molecular weight proteins between

1000-25000 Da. The molecular weight of PAGs is protein specific; however, they are heavier proteins with PAG-1, PAG-6, and PAG-17 weighting approximately 75, 66, and 56 kDa, respectively (Klisch et al., 2005).

The monoclonal antibodies used in the present experiment to analyze PAG concentrations were made in 2015 from the same pure monoclonal antibodies stock used by Green et al. (2005). Monoclonal antibodies managed at constant temperatures can be stored for long periods of times without losing or altering its binding capacity (Goding, 1996). Physical alterations were seeing between L4 monoclonal antibodies batches; however, overall antigen-antibody binding activity was not affected on the three monoclonal antibodies. Indicating that frequency of use does not change monoclonal antibodies capacity when preventing any external contamination that can alter its function.

4.6. Conclusion

Different methods of sample handling can alter PAG concentrations results in an in-house sandwich ELISA; however, plasma or serum samples can be used to effectively diagnose pregnancy on day 28 of gestation in multiparous beef cows. Due to the sensitive association between PAG levels and embryonic loss, further experiments should investigate an influence in predicting embryonic mortality between plasma and serum PAG concentrations. Either plasma or serum samples can be used after short or long periods of incubation prior to centrifugation without altering PAG level results. However, serum concentrations are affected by repeated freeze-thaw cycles, rendering plasma PAG concentration yields more reliable for an in-house sandwiched ELISA.

Ultimately, monoclonal antibodies can be used for long periods of time and with high frequency without altering its antigenicity if external contaminants are avoided.

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5. CONCLUSIONS

One suggested function of pregnancy associated glycoproteins (PAG) is being a marker of placental function; however, they appear to not be related to embryonic-fetal size or growth throughout gestation, nor are they associated with birthweights. Although there were some instances of a relationship being detected, the correlations lacked statistical power. Due to the inverse relationship between PAG and embryo-fetal size on particular gestational days, the correlation between PAG and embryo-fetal size and growth should be evaluated when the secretion of PAG and fetal size are increasing. Even though circulating PAG concentrations does not seem to be related to embryonic-fetal size, utilization of either plasma or serum samples does affect its concentrations. Pregnancy associated glycoproteins concentrations are greater in plasma than in serum samples; however, this does not affect the accuracy of pregnancy diagnosis in cattle. Nonetheless, the impact between these changes ought to be studied in conjunction with the ability of PAG to predict embryonic mortality. In addition to greater concentrations, plasma samples appear to yield more reliable results from the in-house sandwich ELISA given that it was not affected by repeated freezing and thawing cycles, whereas the serum samples were affected. Even so, serum samples can still generate consistent results when samples are stored with minimal or no freeze-thaw cycles. Although serum and plasma PAG concentrations differ in concentration and sensitivity to freeze-thaw cycles, neither is affected by different incubation times before centrifugation or with

utilization of frequently used monoclonal antibodies compared to unfrequently used monoclonal antibodies.

Although PAG functions remain unknown, it is clear that they are not driven by embryonic-fetal size and that concentrations can be altered by various handling methods. These interpretations imply that embryonic loss related to lower PAG concentrations is not due to smaller embryos and that there is a need to obtain a generalized collecting and handling methods that will minimize inaccuracies in PAG concentration results.

APPENDIX A

TABLES

Variables	r[1]	*P* - value
CRL 28d vs PAG 28d	-0.07	0.58
CRL 67d vs PAG 67d	0.02	0.89
Birthweight vs PAG 28d	-0.24	0.12
Birthweight vs PAG 67d	-0.11	0.47
Growth 28-67d vs PAG 28d	0.09	0.48

Table A1. Association between embryonic crown to rump length (CRL), embryo growth, and birthweight with pregnancy associated glycoproteins (PAG) on Location 1. 28d: day 28 of gestation; 67d: day 67 of gestation; 28-67d: embryo growth between days 28 and 67 of gestation.
[1] correlation coefficient

Variables	r[1]	*P* - value
CRL 35d vs PAG 35d	0.10	0.43
CRL 53d vs PAG 53d	-0.14	0.25
Growth 35-53d vs PAG 35d	-0.11	0.36

Table A2. Association between embryonic crown to rump length (CRL) and embryo growth with pregnancy associated glycoproteins (PAG) on Location 2. 35d: day 35 of gestation; 53d: day 53 of gestation; 35-53d: embryo growth between days 35 and 53 of gestation.
[1] correlation coefficient

Variables			
PAG Day	**CRL Day**	**r[1]**	***P* - value**
35	35	-0.10	0.49
42	42	-0.05	0.69
49	49	-0.01	0.95
56	56	0.03	0.82
63	63	0.19	0.17
70	70	0.18	0.18
77	77	-0.16	0.26
84	84	0.15	0.39
91	91	0.13	0.48
42	49	0.14	0.41
42	56	0.01	0.96
42	63	0.02	0.89
42	70	0.43	0.01
42	77	-0.07	0.69
42	84	0.22	0.21
42	91	0.19	0.25

Table A3. Association between embryonic CRL and PAGs on Location 3.
[1] correlation coefficient

Variables			
PAG Day	**Growth Days**	**r[1]**	***P* - value**
35	84-91	0.07	0.68
35	77-84	0.22	0.22
35	70-77	-0.29	0.08
35	63-70	0.22	0.19
35	56-63	0.08	0.62
35	49-56	-0.81	0.65
35	42-49	-0.09	0.59
35	35-42	0.35	0.04
35	35-91	0.26	0.12
42	84-91	-0.03	0.86
42	77-84	0.25	0.16
42	70-77	-0.41	0.01
42	63-70	0.37	0.03
42	56-63	0.01	0.93
42	49-56	-0.11	0.54
42	42-49	-0.05	0.78
42	35-42	0.26	0.13
42	35-91	0.24	0.17
42	42-91	0.15	0.39

Table A4. Association between embryonic growth and PAGs on Location 3.
[1] correlation coefficient

Variables			
PAG Days	**CRL Day**	**r[1]**	***P* - value**
35-42	35	0.53	0.001
	42	-0.07	0.67
	49	-0.07	0.69
	56	-0.04	0.81
	63	0.11	0.54
	70	-0.07	0.67
	77	0.03	0.88
	84	-0.15	0.39
	91	0.02	0.91

Table A5. Association between embryonic CRL and differences in PAGs on Location 3.

APPENDIX B

FIGURES

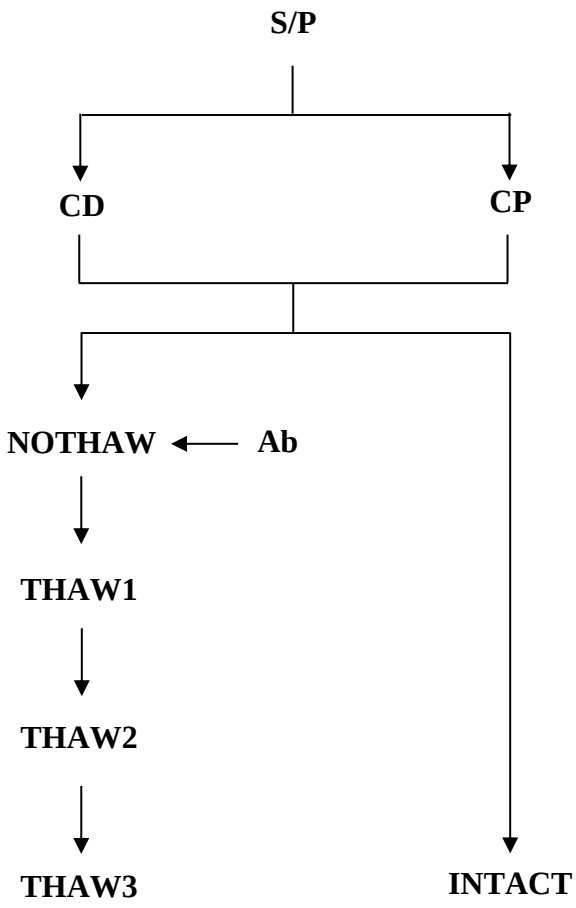

Figure B1. Diagram of procedures. S/P: serum or plasma sample; CD: sample centrifuged on same day of collection; CP: sample centrifuged one day post-collection; NOTHAW: sample analyzed without any freezing processes; THAW1: sample analyzed with one freeze-thaw cycle; THAW2: sample analyzed with two freeze-thaw cycles; THAW3: sample analyzed with three freeze-thaw cycles; INTACT: sample stored and analyzed one-week after day of collection along with THAW3; Ab: sample analyzed with frequently used monoclonal antibodies and unfrequently used monoclonal antibodies.

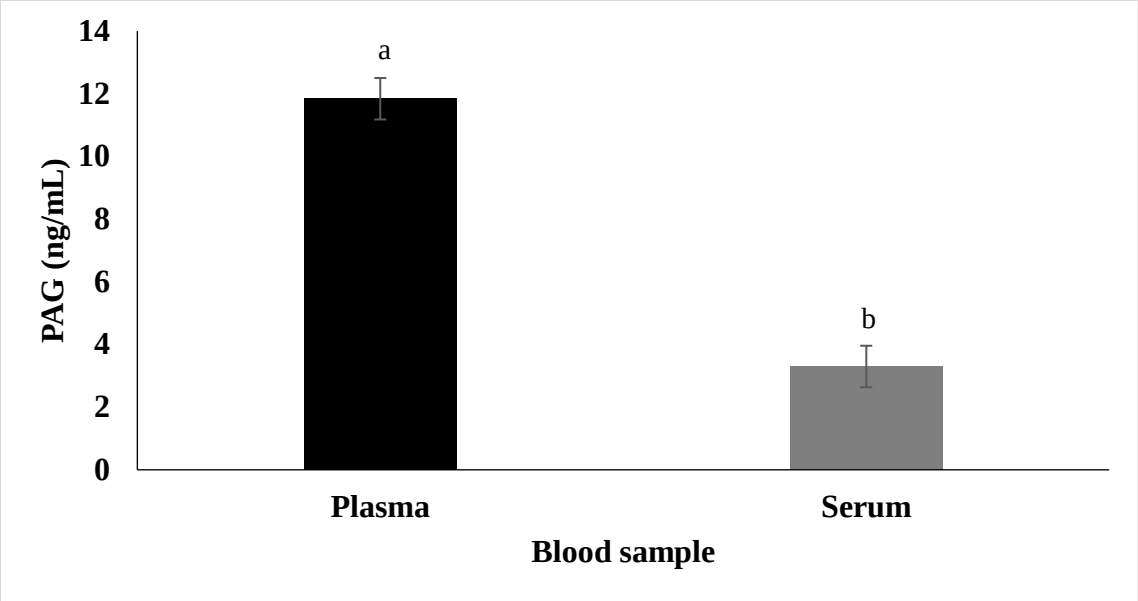

Figure B2. Effects of type of blood sample on pregnancy associated glycoproteins (PAG) concentrations.
[a,b] Significant least square means difference (P < 0.05)

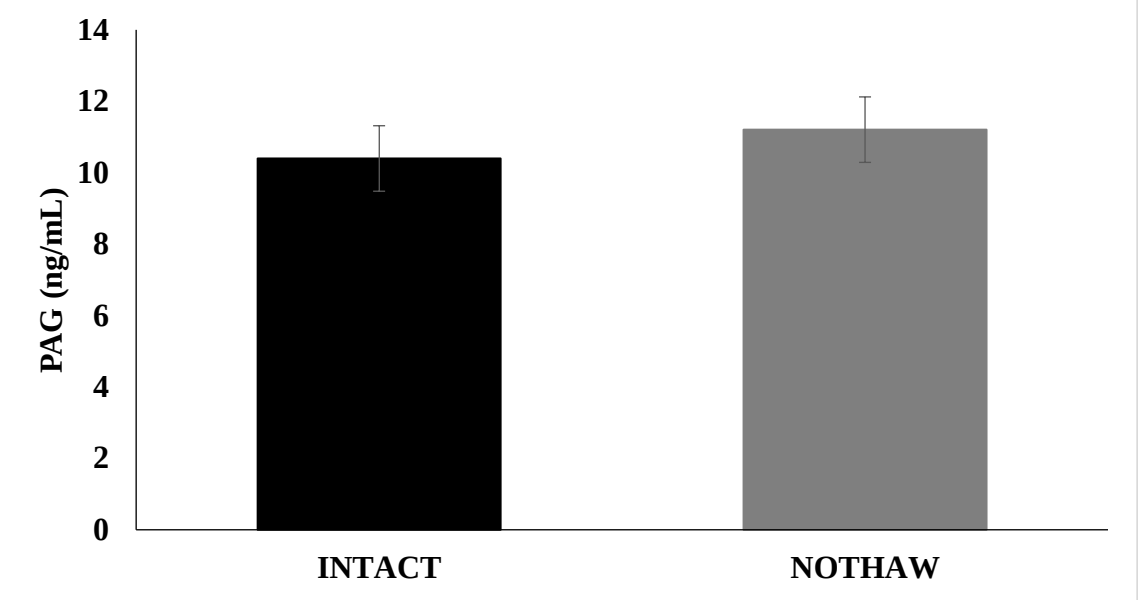

Figure B3. Difference in pregnancy associated glycoproteins (PAG) concentrations between NOTHAW and INTACT sample of plasma samples.
[a,b] Significant least square means difference (P < 0.05)

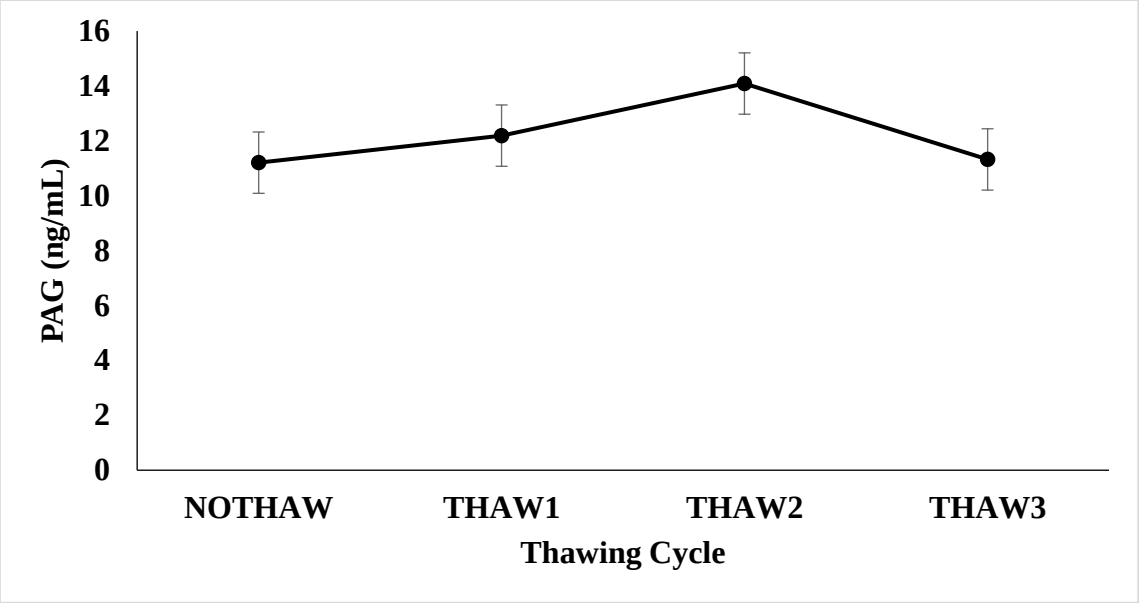

Figure B4. Effects of repeated freeze-thaw cycles on plasma samples on pregnancy associated glycoproteins (PAG) concentrations.
[a,b] Significant least square means difference ($P < 0.05$)

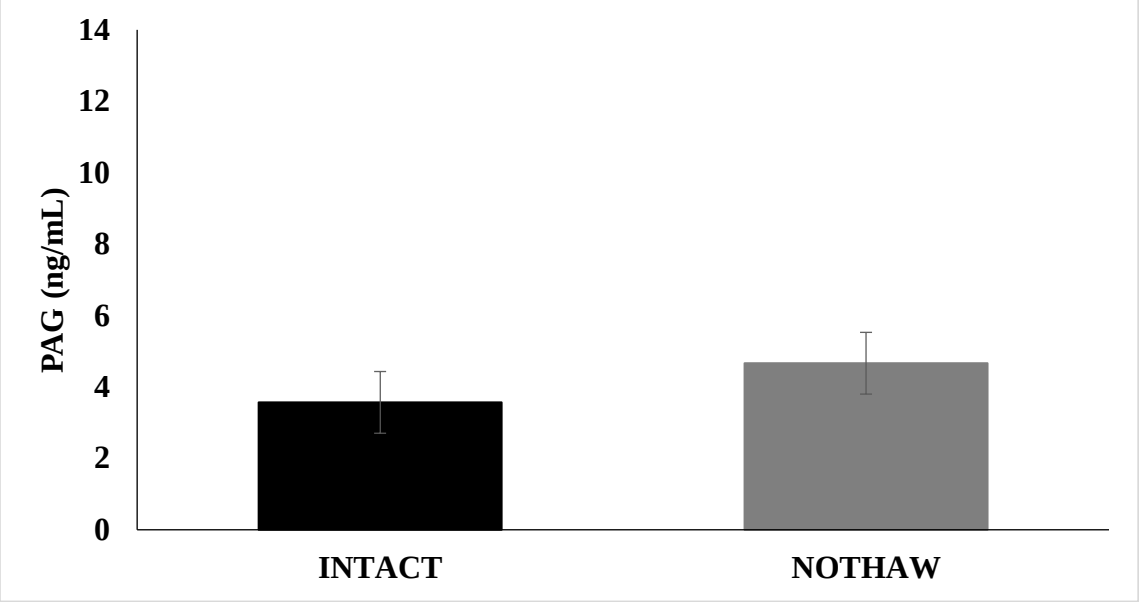

Figure B5. Difference in pregnancy associated glycoproteins (PAG) concentrations between NOTHAW and INTACT sample of serum samples.
[a,b] Significant least square means difference ($P < 0.05$)

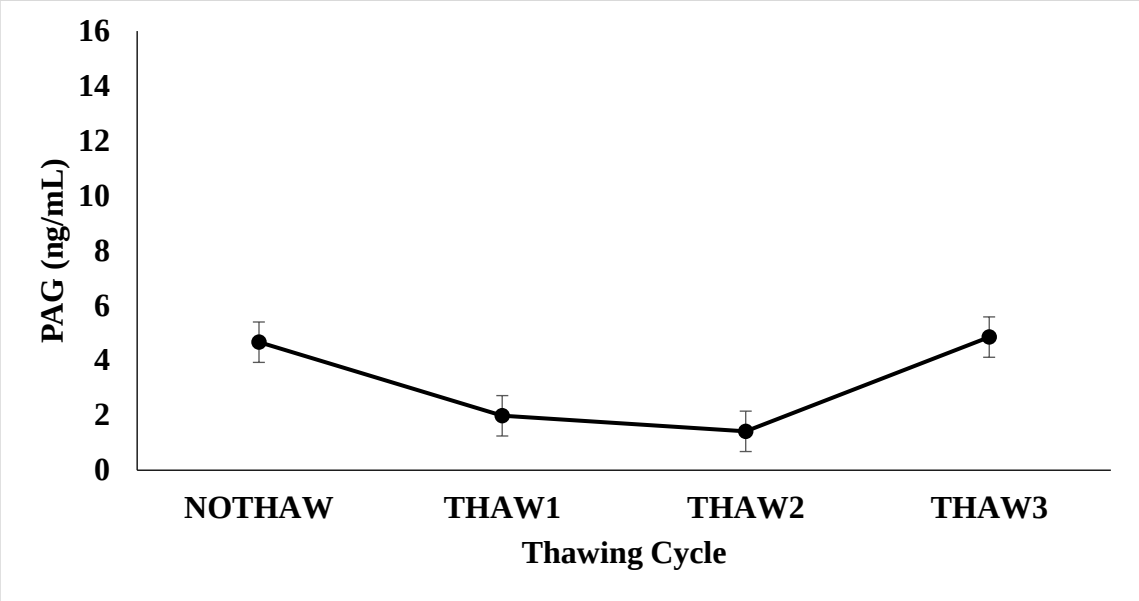

Figure B6. Effects of repeated freeze-thaw cycles on serum samples on pregnancy associated glycoproteins (PAG) concentrations.
[a,b] Significant least square means difference ($P < 0.05$)

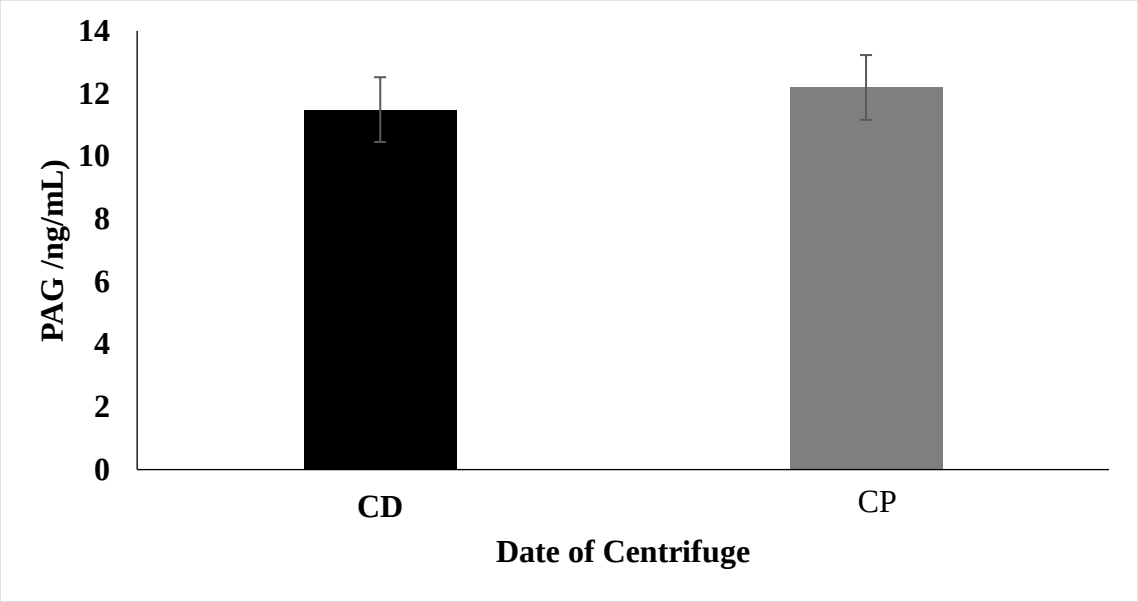

Figure B7. Effects of centrifugation on the same day (CD) or a day post-collection (CP) on plasma samples on pregnancy associated glycoproteins (PAG) concentrations

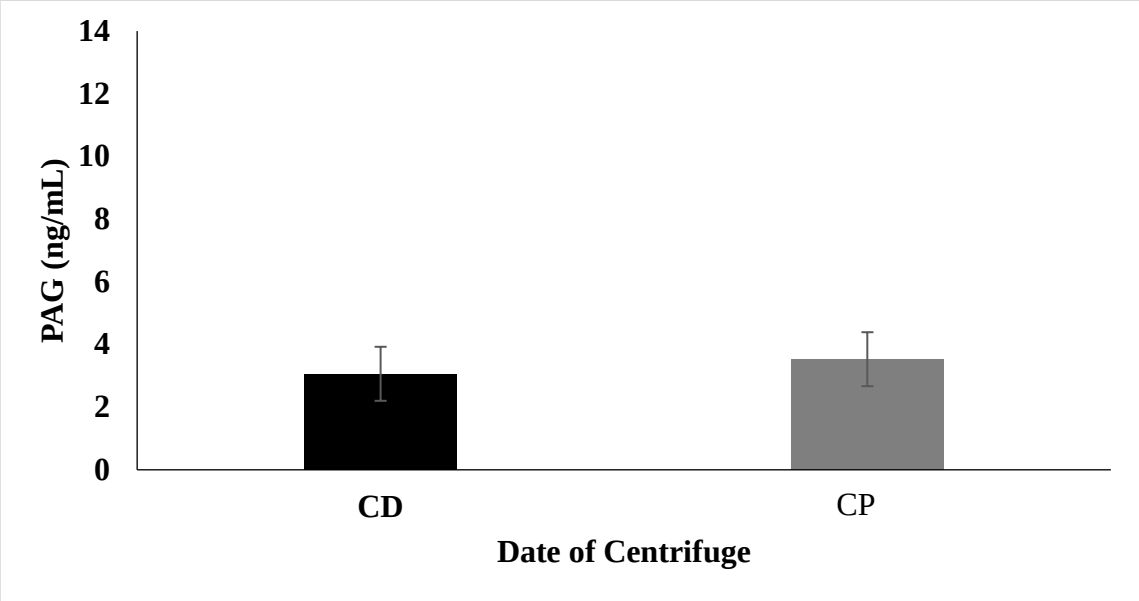

Figure B8. Effects of centrifugation on the same day (CD) or a day post-collection (CP) on serum samples on pregnancy associated glycoproteins (PAG) concentrations.

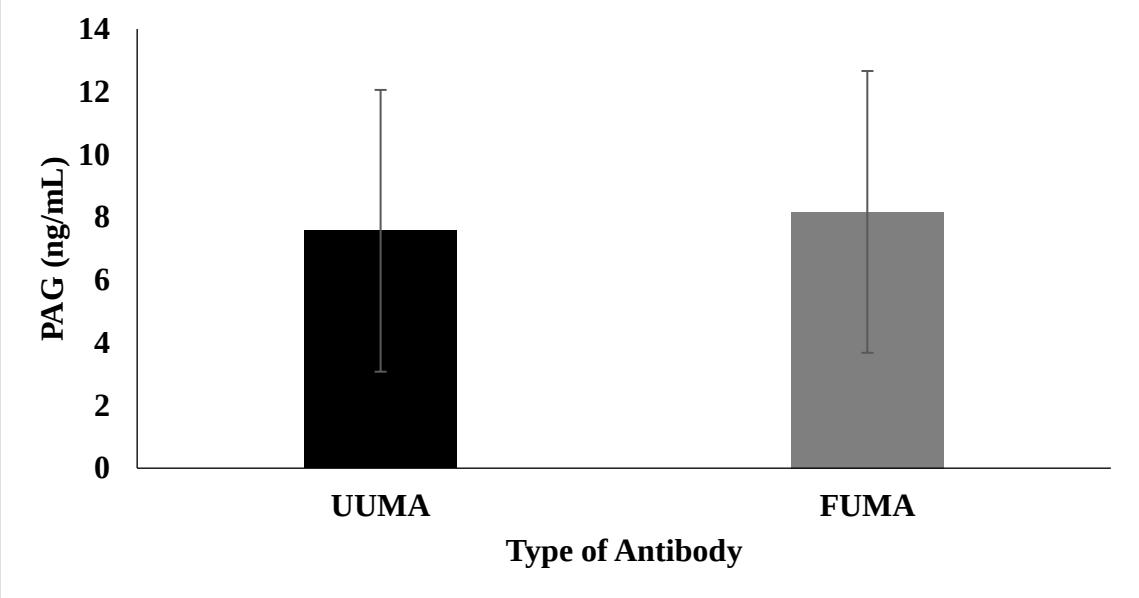

Figure B9. Effects of antibody usage between unfrequently used antibodies (UUMA) and frequently used antibodies (FUMA) on pooled samples of pregnant cows on pregnancy associated glycoproteins (PAG) concentrations.