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Pregnancy associated glycoproteins (PAGs) and pregnancy loss in high vs sub fertility heifers

S.T. Reese [a], T.W. Geary [b], G.A. Franco [a], J.G.N. Moraes [c], T.E. Spencer [c], K.G. Pohler [a, *]

[a] Department of Animal Science, Texas A&M University, College Station, TX, USA
[b] USDA-ARS, Fort Keogh Livestock and Range Research Laboratory, Miles City, MT, USA
[c] Division of Animal Sciences, University of Missouri, Columbia, MO, USA

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ABSTRACT

Reproductive inefficiency and infertility are major financial burdens to domestic livestock. Variables associated with these reproductive losses during early gestation include contributions from the oocyte, uterus, sperm, embryo and placenta. Bovine pregnancy associated glycoproteins (PAG) are produced by the binucleate cells of the ruminant placenta and can be used to diagnose pregnancy. Increased circulating concentrations of PAG early in gestation have been correlated with pregnancy success and decreased concentrations are predictive of impending embryonic mortality in both beef and dairy cattle. The objectives of the current study were to determine whether: 1) heifer fertility status is associated with circulating concentrations of PAG and pregnancy loss; and 2) PAG concentrations within the same animal are repeatable across multiple pregnancies. We hypothesized maternal PAG concentrations would be increased in high fertility compared to subfertile heifers but not repeatable across subsequent pregnancies in the same heifer. Serial embryo transfer (ET; n = 4 rounds) was used to classify predominately Angus heifers (n = 92) as highly fertile (HF = 30; 100% pregnancy success) or subfertile (SF = 62; average = 33%; range = 25–75% pregnancy success) based on day 28 ultrasound diagnosis. Blood samples were collected at both day 28 and 44 for quantification of circulating PAG concentrations by an in house PAG ELISA with antibodies raised against early secreted PAGs. Pregnancy was terminated at day 44 of gestation and heifers were allowed 30 days recovery before synchronization for the next ET. Only heifers that were diagnosed pregnant by ultrasound were used in this study (HF: n = 30, SF: n = 62). Serum concentrations of PAGs were not different between HF (5.90 ± 0.27 ng/mL) and SF (5.56 ± 0.31 ng/mL; P = 0.16) heifers at day 28 of gestation nor was there a difference at day 44 of gestation (P = 0.32). Subfertile heifers had increased pregnancy loss between days 28 and 44 of gestation. Based on odds ratio analysis, SF heifers had a 2.41 times chance to undergo pregnancy loss between day 28–44 compared to HF heifers (P < 0.05). There was no correlation (P > 0.05) in maternal circulating concentrations of PAG between pregnancies on day 28 or 44 of gestation in samples obtained from HF heifers. In summary, circulating concentrations of PAG are not different between HF and SF heifers; however, HF classified heifers have decreased pregnancy loss between days 28 and 44 of gestation.

1. Introduction

Subfertility is a pervasive problem that significantly decreases reproductive efficiency in cattle production systems. Subfertile heifers may be able to successfully establish pregnancy but are often unable to maintain it, undergoing pregnancy loss before the end of gestation [1]. Pregnancy loss may occur at any point during gestation; however, embryonic and fetal mortality decreases as pregnancy progresses [2–4]. Day 30 pregnancy rates in beef heifers bred by artificial insemination are often reported between 55 and 75% [3,5–8] resulting in a 25–45% failure. Reproductive inefficiency in cattle may be associated with multiple variables that include contributions from the oocyte, sperm, embryo, ovary, uterus and the placenta. Research to distinguish characteristics associated with differential fertility have identified associations with reproductive tract score and size, antral follicle count, uterine

* Corresponding author. Department of Animal Science, College Station, TX, 7784, USA
E-mail address: kpohler@tamu.edu (K.G. Pohler).

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environment and maternal/paternal genetics [9–12]. Late embryonic or early fetal mortality, defined as pregnancy loss occurring between days 28 and 60 of gestation, has been less explored and characterized. While less prevalent than early embryonic mortality before day 28 of gestation, late embryonic mortality has been reported from 8 to 15% [13–18]; however, the use of reproductive technologies such as artificial insemination, embryo transfer, and in vitro produced embryos can increase this loss [4,19–22]. Cows undergoing embryo transfer from in vitro produced (IVP) embryos tend to have increased late embryonic mortality compared to in vivo produced embryos or natural service [23–26]. The exact causes or mechanisms associated with these increased reproductive failures remain unknown. Placental abnormalities have been hypothesized as potential causes of late embryonic/early fetal mortality in cattle, specifically in pregnancies resulting from assisted reproductive technologies including IVP embryos and somatic cell nuclear transfer [24,27]. In vitro produced embryos have pregnancies with increased abnormalities in placentome formation, angiogenesis, and allantoic membrane development, which may contribute to increased pregnancy loss observed between day 30 and 90 of gestation [23,24,28–31].

Pregnancy associated glycoproteins (PAG), a family of glycoproteins produced by binucleate cells of the ruminant placenta, can be used to diagnosis pregnancy and may have biological roles in pregnancy maintenance and potentially placental function [13,14,32–34]. Circulating concentrations of PAG from days 28–31 of gestation are accurate measures of pregnancy and pregnancy success in cattle [14,16,35,36]. Thus, the objectives of the current study were to determine whether: 1) heifer fertility classification is associated with circulating concentrations of PAG and pregnancy loss; and 2) PAG concentrations within the same heifer are repeatable across multiple pregnancies. Our hypothesis was that maternal PAG concentrations would be increased in high fertility (HF) compared to subfertility heifers (SF). Furthermore, we hypothesized that concentration of PAGs would not be repeatable across multiple pregnancies in a single animal.

2. Materials and methods

2.1. Animals

All protocols and procedures were approved by Fort Keogh Livestock and Range Research Laboratory (LARRL) Animal Care and Use Committee. Study 1 of Geary et al. [1] describes partial experimental design, embryo production and management of animals in the current study. This study focuses on all heifers included in the larger study with an emphasis on pregnancy loss. Crossbred, predominantly Angus beef heifers (n = 265) were enrolled in a serial embryo transfer (ET) study at the LARRL in Miles City, MT.

2.2. Embryo transfer

Estrus synchronization protocol began when the heifers were approximately 14 months old and weighed 368 ± 2.8 kg. The experiment involved 4 rounds of ET occurring between August 2012 and May 2013 (Fig. 1). During each round of ET, estrus/ovulation was synchronized in heifers using PG- 6d- CIDR protocol ($PGF_{2\alpha}$- 6 days-controlled intravaginal drug releasing device) where $PGF_{2\alpha}$ (Lutalyse, Zoetis Animal Health, Troy Hills, NJ) was administered on day −12. At day −9, GnRH (100 μg intramuscular, Factrel; Zoetis Animal Health, Troy Hills, NJ) was administered and a CIDR (EAZI-BREED CIDR; Zoetis Animal Health, Troy Hills, NJ) inserted. Following CIDR removal on day −3, estrus detection patches (Estrotect; Rockway, Inc. Spring Valley, WI) were applied and heifers were treated with $PGF_{2\alpha}$. Expected estrus was day 0. Heifers were evaluated 3 times daily for estrus activity from day −2 to 0. Animals that did not show signs of estrus received a second dose of GnRH on day 0. Heifers were evaluated on day 7 using a transrectal ultrasound (Aloka SSD 3500 V with a 7.5 MHz convex transducer) to determine presence and location of a CL. Heifers with a CL received a high quality (grade 1) fresh in vitro produced blastocyst or expanded blastocyst stage embryo. Embryos were produced at the University of Florida as described in Geary et al. [1] using oocytes from undefined, abattoir derived ovaries, and high fertility semen from three pooled bulls was used to fertilize oocytes. Ultrasound pregnancy diagnosis occurred on day 28 of gestation and was confirmed on day 44 based on presence of a fetal heartbeat to detect late embryonic/early fetal survival or loss. Blood samples were collected from the coccygeal vein via 10 mL vacutainer (BD Vacutainer, Becton Dickinson and Company, Franklin Lakes, NJ) on day 28 and 44 of gestation for PAG concentration analysis. Blood was allowed to clot at room temperature for 1 h before being stored at 4 °C for 24 h. Following centrifugation of clotted blood samples at 1200×*g* for 25 min, serum was collected and stored at −20 °C. At day 44, remaining pregnancies were terminated with $PGF_{2\alpha}$ and heifers were allowed a minimum of 30 days for recovery before resynchronization began.

Heifers used in this study must have presented a CL on day 7 and received an embryo in at least 3 rounds (n = 265 total; ET by round: 1 = 261; 2 = 258; 3 = 251; 4 = 250). Heifers were classified as highly fertile (HF; 100% pregnancy success) if they maintained pregnancy each time they received an embryo until the first pregnancy diagnosis (day 28). Heifers were designated subfertile (SF; average = 33%; range = 25–75% pregnancy success from 3 to 4 rounds of ET) if they received an embryo in at least 3 rounds and diagnosed pregnant by ultrasound at day 28. Heifers that did not maintain any pregnancy until day 28 after receiving embryos in each round were classified as infertile (IF; 0% pregnancy success; not used in this study). Only heifers that were diagnosed pregnant by ultrasound at least one time at day 28 (HF: n = 30, SF: n = 62) were used in this study. Pregnancy loss was classified as heifers with a positive pregnancy diagnosis determined by ultrasound on day 28 of gestation, followed by a negative diagnosis based on lack of fetal heartbeat at day 44.

2.3. Assay

Serum concentrations of PAG were quantified with in house PAG ELISAs using antibodies raised against PAGs expressed early in gestation [14,16,37] with a sensitivity of 0.28 ng/mL. All samples were run in duplicate. Each plate had a serial dilution protein standard, non-pregnant pooled cow serum controls and third trimester pregnancy pooled cow serum controls. Interassay and intraassay CV's were 9.78% and 5.26%, respectively. Serum concentrations of progesterone were quantified by RIA using a double antibody RIA kit (MP Biomedicals, Costa Mesa, CA) as validated by Pohler et al. [14] with a sensitivity of 0.2 ng/mL. Interassay and intraassay CV's were 9.44% and 8.90%, respectively.

2.4. Statistical analysis

Chi square test in SAS PROC FREQ was used for analyzing pregnancy rates and late embryonic mortality between rounds (SAS 9.4, SAS Institute). One-way ANOVA using PROC GLM was used to determine differences between serum PAG concentrations in day 28 and day 44 samples. To determine repeatability of circulating PAG concentrations across multiple pregnancies, repeated measures with a model that included embryo transfer round as a fixed effect using a correlation structure of autoregressive to test correlations over time was utilized. Frequency of pregnancy loss was

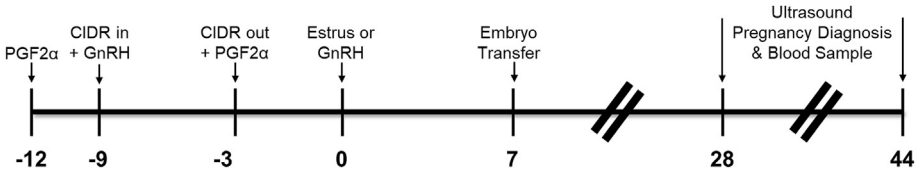

Fig. 1. Experimental design of serial embryo transfer. Heifers were synchronized using a prostaglandin and 6-day CIDR protocol. Embryos were transferred on day 7 after estrus or GnRH treatment. Blood samples and pregnancy diagnosis occurred on day 28 and 44. Serial ET was completed by repeating the process 4 times with at least 30 days of rest between pregnancy termination and initiation of the next estrus synchronization protocol.

compared between heifer fertility classification (HF vs SF) using odds ratio (FREQ procedure, SAS 9.4, Institute Inc., Cary, NC). Probability for prediction of pregnancy maintenance by circulating PAG concentration was determined according to the following equation: Probability = ($e^{\text{logistic equation}}$)/(1 + $e^{\text{logistic equation}}$) using GENMOD procedure (SAS 9.4, Institute Inc., Cary, NC). Significance was set at $P \leq 0.05$, and tendencies were determined if $P > 0.05$ and $P \leq 0.10$.

3. Results

3.1. Pregnancy diagnosis and pregnancy loss

Overall pregnancy rates, including both HF and SF groups, from ET of IVP embryos at day 28 and 44 were 55.5% and 47.6%, respectively. While pregnancy rates in individual rounds of ET ranged from 51% to 59% at day 28 of gestation across all heifers, there was no difference in pregnancy rate between rounds ($P = 0.67$). As expected, SF heifers had decreased pregnancy rates compared to high fertility heifers which had positive pregnancy diagnosis at day 28 in all 4 rounds of ET (HF = 100% pregnancy rate). Pregnancy rate by round of SF heifers reached a high of 42% and a low of 30% (mean 34%); however, there was no difference in SF pregnancy rate by round ($P = 0.46$).

Incidence of pregnancy loss, defined as positive pregnancy diagnosis on day 28 followed by absence of a viable fetal heartbeat at the day 44 pregnancy diagnosis was 22.5% across all rounds and fertility classifications. Overall, HF heifers experienced less ($P = 0.04$) pregnancy loss (15.8%) compared to SF heifers (31.1%). Pregnancy loss was similar in all rounds except round 2 where both groups had increased ($P < 0.001$) pregnancy loss (Table 1). Based on odds ratio analysis, SF heifers were 2.41 ($P < 0.01$; 1.2–4.7; 95% CI) times more likely to experience pregnancy loss from days 28–44 of gestation as compared to HF heifers. Across all rounds, 6 heifers experienced multiple pregnancy loss events (HF = 2 and SF = 4) and the remaining heifers only lost pregnancy a single time (HF = 14 and SF = 20).

3.2. Circulating concentration of pregnancy associated glycoproteins and progesterone

Serum concentration of PAG in successful pregnancies were similar between pregnant HF and SF heifers on day 28 ($P = 0.15$) and 44 ($P = 0.32$; Fig. 2). Concentrations of PAG were decreased ($P < 0.001$) at day 44 compared to day 28 in both HF and SF heifers (Fig. 2), which was expected based on normal secretion patterns of PAG production in early gestation. Additionally, ET round did not influence the average circulating concentration of PAG in successful pregnancies of HF and SF heifers ($P = 0.15$; data not shown). Across both HF and SF heifers, those that experienced pregnancy loss between day 28 and 44 had decreased ($P = 0.02$) circulating PAG concentrations (4.81 ± 0.33 ng/mL) at day 28 compared to heifers that maintained pregnancy (5.74 ± 0.18 ng/mL; Fig. 3). There was a tendency ($P = 0.06$) for HF heifers experiencing pregnancy loss to have decreased day 28 PAG concentrations, but no difference in day 28 PAG concentration among SF heifers that experienced pregnancy loss ($P = 0.22$; Fig. 3). Furthermore, as day 28 circulating PAG concentration increased, there was an increased probability of pregnancy maintenance until day 44 of gestation ($P = 0.02$; Fig. 4). For every 1 ng/mL increase in circulating concentration of PAG at day 28, there was a 7.2% increase in the probability of pregnancy maintenance, whereas for every 1 ng/mL decrease in PAG there was a 20% increase in the probability of pregnancy loss.

In order to assess the repeatability of circulating PAG concentrations in individual heifers, PAG concentrations of successful pregnancies from HF heifers of each round were compared at both day 28 and day 44. Based on four rounds of ET, there was no correlation ($P = 0.61$) in circulating PAG concentrations between pregnancies in the same HF heifer on day 28 or day 44 of gestation.

Circulating progesterone concentrations did not differ between HF and SF heifers at day 7 (4.51 ± 0.16 ng/mL vs 4.54 ± 0.18 ng/mL; $P = 0.88$), 28 (5.12 ± 0.37 ng/mL vs 5.25 ± 0.43 ng/mL; $P = 0.82$) or 44 of gestation (5.36 ± 0.46 ng/mL vs 5.77 ± 0.45 ng/mL; $P = 0.33$). Additionally, day 28 progesterone concentrations did not differ ($P = 0.26$) between heifers maintaining a pregnancy to day 44 (5.35 ± 0.31 ng/mL) or heifers experiencing pregnancy loss (4.51 ± 0.61 ng/mL).

Table 1
Pregnancy loss between days 28 and 44 of gestation by fertility classification.

	Pregnancy loss between d 28 and 44			
	High Fertility	Subfertility	SEM	P-value
Round 1	10.3% (3/29)	11.5% (3/26)	6.25	0.73
Round 2	24.1% (7/29)	65.0% (13/20)	9.69	0.01
Round 3	17.9% (5/28)	28.0% (7/25)	6.48	0.01
Round 4	10.7% (3/28)	26.3% (5/19)	8.03	0.14
Overall	**15.8%**	**31.1%**	**4.13**	**0.03**

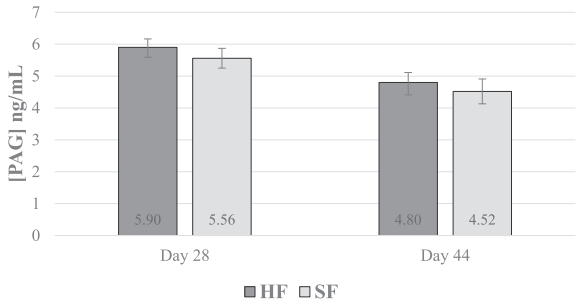

Fig. 2. Circulating PAG concentrations of fertility groups by day of gestation. Circulating PAG concentrations (ng/mL) were not significantly different between successful pregnancies in high fertility (HF) and subfertile (SF) heifers at day 28 (5.90 ± 0.27 vs. 5. ± 0.31; $P = 0.15$) or day 44 (4.80 ± 0.31 vs 4.52 ± 0.39; $P = 0.32$) of gestation.

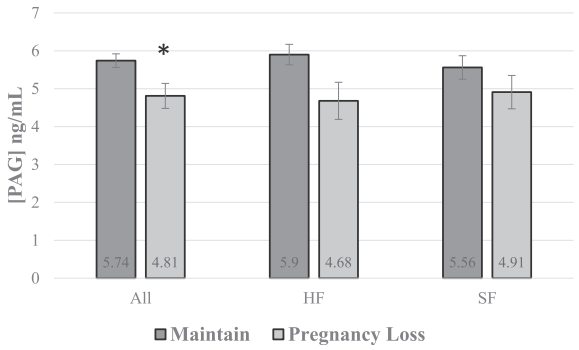

Fig. 3. Day 28 PAG concentration by pregnancy outcome. All high fertile (HF) and subfertile (SF) heifers maintaining pregnancy from day 28–44 had increased circulating PAG concentrations at day 28 (5.74 ± 0.18 ng/mL) compared to heifers that experienced pregnancy loss (4.81 ± 0.33 ng/mL; P = 0.02) regardless of fertility classification. Among HF heifers, PAG concentration tended to be decreased on day 28 for those that experienced pregnancy loss by day 44 (5.90 ± 0.27 vs 4.68 ± 0.49, P = 0.06). Among SF heifers, PAG concentration did not differ on d 28 for those that experienced pregnancy loss by day 44 (5.56 ± 0.31 ng/mL vs 4.91 ± 0.44, P = 0.22).

4. Discussion

The present study found that subfertile classified heifers have increased pregnancy loss compared to high fertility classified heifers but circulating concentrations of PAG in successful pregnancies do not differ in either group. This compliments findings from Geary et al. (2016) which demonstrated that subfertile heifers also experienced greater pregnancy loss during early embryonic development between days 7–28 of gestation. Heritability of many reproductive traits in females is low which has made selection for high fertility animals difficult [38]. Despite advances in genomic selection including identification of genes and loci associated with pregnancy establishment and endometrial receptivity [39], identifying markers of fertility remains a priority. Heifers in both HF and SF groups came from similar genetic backgrounds and had similar circulating progesterone on day 7 at ET in the current study, as well as similar profiles following ovulation and AI until day 36 of gestation [1]. While uterine deficiencies that impact fertility of a single ovulation have been identified including regulation of steroid hormone receptors in the endometrium [40,41], minimal differences in endometrial gene expression were observed between these HF and SF heifers during early gestation (day 14 or 17) [35].

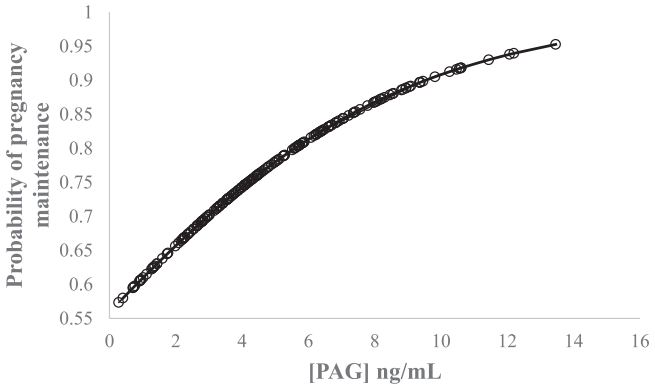

Fig. 4. Probability of pregnancy maintenance increases as circulating PAG concentrations increase at day 28 of gestation. Increased circulating concentrations of PAG on day 28 significantly increased (P = 0.02) the probability of pregnancy maintenance until day 44 of gestation. The likelihood of pregnancy maintenance after embryo transfer between days 28 and 44 increases 7.2% for every 1 ng/mL increase in circulating PAG concentration.

However, the endometrial response to pregnancy was substantially altered in SF heifers [42]. Although heifers were assigned to a fertility classification based on day 28 pregnancy rates, pregnancy loss after day 28 was significantly increased in SF heifers compared to HF heifers. This suggests that animals with subfertility will have decreased reproductive efficiency compared to high fertility herds even after a similar initial pregnancy diagnosis.

The incidence of late embryonic/early fetal mortality in cattle varies widely from 3% to greater than 40% and is influenced by factors including production status, environmental stressors, and genetics [13–15,17,19,43–46]. Circulating PAG concentrations has been used as a predictive measure of late embryonic/early fetal mortality by multiple groups and as a marker of placental function [14,47–51]. Decreased circulating concentrations of PAGs early in gestation, specifically between days 28–30, have been reflective of embryonic death or pregnancy failure with the half-life of PAGs during this period being around ~35 h [13]. Physiologically, pregnancy loss after day 28 of gestation coincides with a period of active placentation marked by extensive endometrium remodeling, binucleate cell migration and changes in PAG expression production [13,52]. Due to increased pregnancy loss in the subfertile group, we hypothesized that subfertile animals would have decreased circulating PAG concentrations. While day 28 PAG concentrations were decreased for all heifers that experienced pregnancy loss before day 44, and tended to be decreased among HF heifers, they were not decreased among SF heifers in the current study. Moraes et al. [42] reported that the expression of PAG12, one of over 20 PAG genes in the bovine genome, was increased 2.93-fold in HF compared to SF conceptuses at day 17 of gestation. Although there was no difference in circulating PAG concentrations between successful pregnancies that were maintained until day 44 of gestation in HF and SF heifers, SF heifers were more than twice as likely to undergo pregnancy loss. Similar to previous reports in cows and heifers of both *Bos indicus* and *Bos taurus* origin [13,15,49,50], circulating PAG concentrations were decreased in heifers experiencing pregnancy loss regardless of fertility classification. In this study, a decrease in PAG concentration of 1 ng/mL increased the chance of pregnancy loss in both HF and SF heifers by 20%.

Using IVP embryos for embryo transfer results in decreased pregnancy rates and increased pregnancy loss which may have played a role in the increased embryonic mortality that was observed in particular rounds of this present study [23,24,53]. Additionally, some studies report differences in PAG concentration between pregnancies from IVP produced embryos compared to in vivo produced pregnancies [54], while others find no difference [50,55]. Differences in PAG concentration from pregnancies of IVP embryos may be impacted by the higher frequency of abnormalities which accompany the placental development of these pregnancies [24,29]. Pregnancies from SF heifers may be more vulnerable to these abnormalities and subsequent mortality throughout gestation. Fertility classified heifers had similar embryo recovery rates at day 14 of gestation yet SF heifers had lower pregnancy rates at day 28 which suggests a significant portion of pregnancy loss occurs at or after maternal recognition of pregnancy [1]. Using a subset of these fertility classified heifers, Moraes et al. (2018) reported that pregnancy loss in SF heifers is increased from day 17–28 due to insufficient conceptus - endometrial interactions based on the findings that at day 17 HF and SF had similar pregnancy rates; however, the heifers had previously been documented as having different day 28 pregnancy outcomes. Embryonic mortality soon after maternal recognition of pregnancy may be a more significant period of pregnancy loss than historically acknowledged, specifically for low fertility animals [16,56]. In a similar model, McMillen and Donnison [56] suggested that low fertility heifers had

increased embryonic mortality between day 25 and 35 compared to high fertility heifers. Overall, they reported 25% of heifers that were not pregnant at day 35 failed to return to estrus by day 25 of gestation indicating pregnancy loss between day 25 and 35. However, when separated by fertility classification, the incidence of embryo mortality during this period was 56% in low fertility heifers compared to 8% in high fertility heifers [56]. Assessing pregnancy loss during this period is challenging due to the difficulty of establishing a positive pregnancy diagnosis prior to day 27 of gestation. This relationship of increased pregnancy failure in SF animals may persist into late embryonic development as indicated by the increased rates of late embryonic mortality in SF heifers reported here.

Due to the low number of pregnancies established by SF heifers, the likelihood of multiple late embryonic failures was unable to be assessed within the same heifer. Overall, 6 heifers underwent pregnancy loss twice in the study which included heifers in the HF and SF groups. However, this model provided the opportunity to compare multiple pregnancies in high fertility classified females. Circulating concentration of PAGs from one pregnancy to the next in a single animal has not yet been reported in the literature. In the current study, circulating PAG concentration was not repeatable across multiple pregnancies in a single HF heifer. This is consistent with the hypothesis that embryo or perhaps paternal-based factors have a strong influence on circulating PAG concentrations. In a recently reported reciprocal embryo transfer project using *Bos taurus* and *Bos indicus* recipients and embryos, there was no effect of recipient; however, the embryo genotype (*Bos indicus* or *Bos taurus*) had a significant impact on circulating PAG concentrations [57]. Additionally, a major paternal influence on circulating PAG concentrations early in gestation has been reported [15,17]. Franco et al. (2018) reported higher PAG concentration in *Bos taurus* sired pregnancies compared to *Bos indicus* sired pregnancies in Nelore cows which provides further evidence for embryonic influence affecting circulating PAG concentrations early in gestation. In the current study, pooled semen was used for fertilization so potential differences due to sire could not be addressed. However, sire influence may explain some of the variation seen between individual pregnancies in a single heifer. Further research is needed to elucidate the factors which control PAG production and secretion and the physiological differences in high and sub fertile cattle to improve reproductive efficiency. This study, combined with previous data from the model, indicates the subfertile phenotype persists beyond early embryonic development and the cumulative process which results in reproductive success is impaired at multiple stages.

5. Conclusion

In summary, pregnant high and subfertile heifers carrying IVF-produced embryos have similar PAG concentrations at days 28 and 44 of gestation. However, PAG concentrations were reduced at day 28 in pregnant heifers that experienced pregnancy loss by day 44 of gestation. Therefore, increases in circulating concentrations of PAG early in gestation are predictive of developing embryo success but not indicative of the dam's fertility status; however, subfertile heifers have a disproportionally high rate of late embryonic/early fetal mortality compared to high fertility heifers. Additionally, maternal PAG concentrations appear to be embryo rather than dam dependent as they were not repeatable over successive pregnancies within dam.

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