Theriogenology 108 (2018) 269–276

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

The ability to predict pregnancy loss in cattle with ELISAs that detect pregnancy associated glycoproteins is antibody dependent

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ARTICLE INFO

Article history:
Received 11 August 2017
Received in revised form
5 December 2017
Accepted 7 December 2017
Available online 14 December 2017

Keywords:
Embryonic mortality
Fetal mortality
Pregnancy associated glycoproteins
Cattle

ABSTRACT

The concentration of circulating pregnancy associated glycoproteins (PAGs) early in pregnancy may serve as markers to predict late embryonic mortality or fetal mortality in cattle. In this study, pregnancies were established in dairy cows, by either fixed-time AI (FTAI) or fixed-time embryo transfer (FTET) with in vitro produced embryos. Circulating PAGs were measured with different combinations of antibodies in either a laboratory-based ELISA or a commercial ELISA. For the in-house ELISA, three monoclonal 'trapping' antibodies (A6, J2, and L4) and two polyclonal 'detection' antisera (antibodies F2 or 45) were used to quantify PAGs in serum from the same cows. The different assays were identified as follows: 'Mix-45' (A6, J2, and L4 with 45), 'Mix-F2' (A6, J2, and L4 with F2), and 'L4-F2': (L4 with F2); the commercial assay was from IDEXX. Ovulation was synchronized and FTAI or FTET was performed on day 0 or 7, respectively. Ultrasound-based diagnosis of pregnancy and serum collections occurred on day 30. The proportion of cows that subsequently experienced pregnancy loss between days 30 and 60 was 23% (43 of 183) and 16% (21 of 131) for the FTAI or FTET groups, respectively. In the FTAI group, mean serum concentration of PAGs detected with Mix-45 was higher in cows that maintained pregnancy (9.2 ± 0.4 ng/ml; mean ± SEM) compared with cows that experienced pregnancy failure (3.9 ± 0.6 ng/ml) between day 30–60 ($P < .001$). However, there was no difference ($P > .69$) in circulating concentrations of PAGs between cows that experienced loss or survival between days 30 and 60 when Mix-F2 or L4-F2 were used in an in-house ELISA. Likewise, a commercial assay also did not result in measurable differences in PAG concentrations between those animals that experienced loss or survival. Following FTET, circulating concentrations of PAGs on day 30 were lower ($P < .001$) in cows that experienced pregnancy failure compared to cows that maintained pregnancy when the Mix-45 and the commercial assay were used, but not with the other antibody combinations. A receiver operating characteristic curve showed that only the Mix-45 antibody combination was predictive (95% accuracy) of pregnancy loss but not the other antibody combinations following FTAI. However, both Mix-45 and the commercial assay were predictive of losses following FTET. In summary, although multiple PAG assay formats have been shown to accurately detect pregnancy, the ability to predict embryo survival during early gestation appears to be antibody dependent.

1. Introduction

Embryonic and fetal mortality is a major cause of reproductive failure in cattle. Pregnancy losses can be categorized in multiple ways, but a simple designation that our group has employed is to define pregnancy losses in cattle based on whether they are occurring early (in the first month of development) or late (after about one month of development) in gestation [1–3]. The losses in the 'late' category can be defined as either late embryonic mortality or fetal mortality depending on when the losses are taking place

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https://doi.org/10.1016/j.theriogenology.2017.12.021
0093-691X/

during the pregnancy. In the current manuscript, the general term 'pregnancy loss' or similar phrasing will be used to describe both embryonic or fetal losses occurring after the first month of pregnancy in cattle.

In cattle, pregnancy loss has been reported to vary from 3 to 42% in beef and dairy cattle [4,5]. To more effectively investigate the physiological mechanisms associated with mortality, a circulating marker that predicts pregnancy loss would be very useful. Pregnancy associated glycoproteins (PAGs) are members of the aspartic proteinase gene family. Many of the bovine PAGs are produced by giant binucleate cells (BNC) in the trophectodermal epithelium [6,7]. These cells are capable of delivering the contents of their secretory granules to the maternal uterine stroma; some of these products, which includes many of the PAGs, can subsequently enter the maternal circulation and can be detected soon after initiation of placentation [8–10]. Many studies have shown that circulating concentrations of PAGs around days 28–31 after insemination are decreased in both *Bos taurus* [1,3,11–14] and *Bos indicus* [2] cattle that subsequently undergo loss of the pregnancy. Conversely, others have reported that PAGs in the maternal circulation are not predictive of pregnancy loss [15]. These apparent discrepancies could be explained by the fact that the PAG family is relatively large (>20 members) and individual PAGs have different expression patterns in cotyledonary tissue during gestation in cattle [16–18].

In 2005, Green et al. developed a PAG sandwich ELISA that made use of both monoclonal (trapping) antibodies and polyclonal (detection) antibodies [10]. The monoclonal antibodies were directed against PAGs produced in cotyledonary tissue during the first 80 days of pregnancy. Recently, Pohler and colleagues used essentially the same assay, differing from the original in the use of a distinct polyclonal detection antibody (Ab45), to detect a difference in serum concentrations of PAGs on day 28 in groups of beef [2,3] and dairy [1] cows that experienced embryonic survival or mortality.

We hypothesized that incorporating antibodies directed against distinct PAGs would result in an ELISA that was capable of detecting differences in circulating concentrations of PAGs in dairy cows likely to undergo pregnancy loss between days 30 and 60 of gestation. Therefore, the objective was to determine if there is a specific monoclonal/polyclonal antibody combination that would also function as accurate a predictor of late embryonic mortality in cattle in the same way as the Mix-45 antibody combination [1,2]. In addition, we examined the efficacy of a commercial PAG assay from IDEXX to assess its capacity for predicting embryonic survival or loss after day 28 of gestation.

2. Materials and methods

2.1. Experiment 1- fixed-time AI (FTAI)

2.1.1. Animals and treatments

Serum samples were obtained from an experiment previously reported by Pohler et al. [1]. All protocols and procedures for animal studies were approved by the local Institutional Animal Care and Use Committee and were based on recommendations in the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (FASS, 1999). Estrus was synchronized in 314 lactating Holstein cows. Estrus was synchronized as follows: On day -11 cows were treated with 2.0 mg (i.m) of estradiol benzoate (EB, 2.0 ml of Estrogin, Farmavet, São Paulo, SP, Brazil); at day -11 they also received an intravaginal progesterone containing insert (CIDR; 1.9 g of P4, Zoetis, São Paulo, Brazil), which was left in place for nine days. On day−4, cows were treated with 25 mg (i.m.) dinoprost tromethamine (PGF2α; 5.0 mL of Lutalyse, Zoetis, Brazil). On day -2, the CIDR was removed and 1.0 mg (i.m.) of estradiol cypionate (0.5 mL of E.C.P., Zoetis, Brazil) was administered. Those animals assigned to the FTAI group were artificially inseminated on day 0.

Cows were housed on three different farms in Brazil. On the first day of the experiment (day-11), cows averaged 147 ± 4.19 days in milk (DIM), yielding 29.5 ± 0.3 kg of milk/day and had a body condition score (BCS) of 2.84 ± 0.02 (on a 1 [emaciated] to 5 [obese] scale [19]. The animals were artificially inseminated on day 0 with frozen-thawed semen from 13 different sires. Diagnosis of pregnancy was performed on days 30 or 31 (collectively referred to as 'day 30') and day 60 following insemination. Only cows that had a single viable embryo (i.e. a heartbeat detected by ultrasonography) were used in the study. There were 183 pregnant cows (90 primiparous cows, 93 multiparous cows). Pregnancy loss was determined between days 30–60 by using real-time ultrasonography; pregnancy loss between day 30 and parturition was also determined.

2.1.2. Ultrasonography

Pregnancy status and embryo viability (as indicated by the detection of heart beat) were examined on day 30 and 60 of gestation and just before parturition by transrectal ultrasonography (Aloka 500 V ultrasound with 5 MHz [MHz] transrectal linear probe). Time of embryonic mortality and fetal loss were determined between day 30 and 60 and between day 30 until parturition, when the embryo/fetus was no longer present.

2.1.3. Blood sample collection

Blood samples were collected via jugular venipuncture (Vacutainer tubes; Fisher Scientific, Pittsburgh, PA) on day 30 of gestation to measure circulating concentration of PAGs. Blood samples were allowed to clot at room temperature for 1 h and stored at 4 °C for 24 h before centrifugation at 2000 × g for 15 min. Serum was collected, and stored at -20 °C until concentrations of PAGs were determined by using a laboratory-produced PAG ELISA as described elsewhere [3,10] or by using a commercial PAG assay (IDEXX, Westbrook, Maine).

2.1.4. Enzyme linked-immunosorbent assay (ELISA)

Development of monoclonal and polyclonal antibodies directed against PAGs was described previously [10]. Circulating concentrations of PAGs were determined by an in-house sandwich ELISA with a mixture of monoclonal antibodies (trapping antibody; A6, J2, and L4) followed by a polyclonal antibody (detection antibody: 'F2' or '45') [10]. The monoclonal antibodies exhibit greatest reactivity toward a relatively small group of known PAGs (e.g. PAGs 4, 6, 9) and the polyclonal antibodies were raised against fractionated/enriched PAGs as described previously [10]. The antigens used to generate the '45' and F2 polyclonal antibodies represented mixtures of PAGs consisting mainly of PAG4 as well as lesser amounts of PAGs 6, 7, 16 and 20. The F2 antigen also contained PAG2. An antigen used to create a detection antibody in the commercial assay consisted of a mixture of PAGs (e.g. PAGs 4, 6, 9, 16, 18 19; described in US Patent no. 7,604,950B2).

The antibody combinations in the in-house ELISA were as follows: Mix-45 (a combination of trapping antibodies A6, J2, and L4 with polyclonal antibody 45), Mix-F2 (trapping antibodies A6, J2, and L4 with polyclonal antibody F2), and L4-F2 (trapping antibody L4 with polyclonal antibody F2). The Mix-45 format was identical to that used in other recent published reports [1–3] and it essentially served as a control assay against which the other combinations were compared. In addition, a commercial PAG ELISA from IDEXX was also employed. Each assay plate included a standard curve (generated from a mixture of purified PAGs), a positive control sample (a pool of sera from day 60 pregnant cows) and a negative control sample (a pool of nonpregnant sera from cows).

Furthermore, both the day 30 and the day 60 serum samples from each cow were on the same ELISA plate. The PAG standard consisted of roughly equal amounts of PAG 4, 6 and 9 as well as trace amounts of PAG 17, 19, 20 and 21. The intra-assay coefficient variation (CV) for the Mix-45, Mix-F2, L4-F2, and commercial PAG ELISA was 4.8, 5.7, 7.0, and 8.0% respectively, and the inter-assay CV was 14.0, 11.7, 16.9, 7.9%, respectively.

2.2. Experiment 2—fixed-time embryo transfer (FTET)

2.2.1. Animals and treatments

The samples analyzed represented a subset of sera that were obtained from an experiment reported previously [1]. All protocols and procedures for animals studies conducted in Brazil conformed to recommendations in the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (FASS, 1999). Pregnant, lactating Girolando cows (~3/4 Holstein − *Bos taurus X Bos indicus;* n = 131) located on Staluzia Farm in Brazil were used for this study. The embryos were produced in vitro (IVF) with frozen-thawed gender-selected semen. All embryos were at the blastocyst stage and only grade 1 (excellent) embryos were used. For animals assigned to the FTET group, recipient cows were synchronized as described for experiment 1. Transfer of IVF-derived embryos into recipients was performed on day 7. Each recipient received a single embryo. Diagnosis of pregnancy and determination of embryonic viability was performed on day 30 and 60, following synchronized ovulation, by real-time ultrasonography. In this experiment, there were 48 primiparous and 83 multiparous cows. The average days in milk (DIM) on the first day of the experiment was 135 ± 4.9, milk production = 18.9 ± 0.4 kg of milk/day, mean cow body condition score (BCS) was 2.86 ± 0.03 (in a 1 [emaciated] to 5 [obese] scale) [19], and the number of times recipients underwent the ET procedure was 2.07 ± 0.15 times. Pregnancy loss was determined between days 30–60 as described for experiment 1.

2.2.2. Blood sample collection and PAG ELISA

Blood samples were collected via venipuncture and processed as described for experiment 1. Circulating concentrations of PAGs were determined in an in-house sandwich ELISA or a commercial ELISA as described for experiment 1. Intra-assay coefficient of variation (CV) for PAG assays for the Mix-45, Mix-F2, L4-F2, and commercial PAG ELISA was 7.9, 5.2, 4.3 and 8.4, respectively and the inter-assay CV for PAG assays was 14.0, 11.7, 16.9 and 8.0, respectively.

2.2.3. Statistical analysis (experiments 1 and 2)

One −way ANOVA (SAS) was used to determine if circulating concentrations of PAGs differed on day 30 between cows that maintained pregnancy compared to cows that did not maintain pregnancy when four antibody combinations (Mix-F2, L4-F2, Mix-45 or a commercial ELISA) were compared. Logistic regression (LOGISTIC procedure in SAS) was used to examine the probability of late embryonic mortality occurring after FTAI (experiment 1) or FTET (experiment 2) based on serum concentrations of PAGs on day 30 of gestation. Receiver operating characteristic curves (ROC; MedCal software) were used to evaluate the in-house ELISA and the commercial ELISA to test the hypothesis that circulating concentrations of PAGs on day 30 of gestation can predict late embryonic mortality in lactating dairy cows. ROC curves were established by plotting the correlation between the specificity and sensitivity at numerous cut-off levels. The predictive accuracy of the diagnosis is explained by the area under the curve (AUC). An AUC of 50% indicates no predictability and refers to the diagonal line at a 45° angle in the ROC. However, an increase in the ACU above 50% reflects an increased accuracy of the test(s).

3. Results

3.1. Experiment 1

On day 30 following FTAI, 183 cows were diagnosed pregnant via transrectal ultrasound. Between days 30–60 of pregnancy 42 (23%) cows experienced mortality and 141 (77%) of the cows maintained pregnancy; an additional 28 pregnancies were lost after day 60 (i.e. failed to deliver a calf at term). All cows had an embryo with a heartbeat on day 30 and mean serum concentration of PAGs, as detected with an in-house ELISA (Mix-45; positive control), were significantly greater ($P < .001$) in animals that maintained pregnancy (mean ± SEM = 9.16 ± 0.44 ng/ml) compared with cows that subsequently experienced embryonic mortality (mean ± SEM = 3.89 ± 0.57 ng/ml) at any point between days 30–60 of gestation (Fig. 1). However, there was no difference in circulating concentrations of PAGs on day 30 between cows that experienced survival of pregnancy compared to pregnancy loss when the Mix-F2, L4-F2, or commercial PAG ELISAs were used. Similar results were found for cows that experienced embryonic mortality between day 30 and parturition (data not shown). Logistic regression was conducted to examine the probability of late embryonic mortality based on PAG concentrations on day 30 after FTAI for cows that experienced embryonic survival or loss between days 30–60 of gestation. When PAGs were quantified with the Mix-45 ELISA, increasing circulating concentrations of PAGs on day 30 were correlated with a decreased probability ($P < .01$) of late embryonic mortality in lactating dairy cows ($P < .01$; Fig. 2). The other PAG antibody combinations and the commercial PAG assay did not reveal a relationship between circulating PAGs and probability of embryonic mortality.

A ROC curve was used to compare the effectiveness of the Mix-F2, L4-F2, and the IDEXX PAG ELISA to the Mix-45 antibody combination for purposes of identifying a circulating concentration of PAGs that would be 95% accurate in detecting late embryonic mortality following FTAI (Fig. 3). An ROC curve is a graphical depiction of the relationship between true positive rate and false positive rate as an increasing cutoff for the PAG test is applied. When the true positive rate and the false positive rate both decrease as the cutoff value is increased this results in a diagonal line through the center of the graph, which means the test is not predictive (50:50 probability; see lines for Mix-F2, L4-F2 and IDEXX assay in Fig. 3). Alternatively, when an ROC curve is deflected to the left of center, the test becomes more useful since it has a relatively high true positive rate and a low false positive rate at a specific cutoff. This scenario was observed for the line representing the Mix-45 assay (Fig. 3). The accuracy of the diagnosis of embryonic mortality can be explained by the area under the curve (AUC) as calculated from the ROC. The closer the AUC is to 1 the more accurate it is; whereas, an AUC of .5 indicates the test is not accurate. The Mix-45 assay had the largest AUC (0.83), for detecting late embryonic mortality between days 30–60 of pregnancy, compared to the L4-F2 (0.54), Mix-F2 (0.50) and the commercial PAG assay (0.51) (Table 1). In addition, utilizing the positive and negative predictive value analysis for the results from the Mix-45 assay, a circulating concentration of PAG >8.43 ng/ml was 95% accurate in predicting embryonic survival and a concentration of PAG <1.62 ng/ml at day 30 of gestation was 95% accurate in predicting pregnancy loss ($P < .001$). The other assays, Mix-F2 ($P < .89$), L4-F2 ($P < .46$), and the commercial PAG assay ($P < .71$) were not able to accurately predict late embryonic or fetal mortality (Table 1). When cows experienced embryonic mortality between day 30 to parturition, only the Mix-45 assay was accurate in diagnosing embryonic

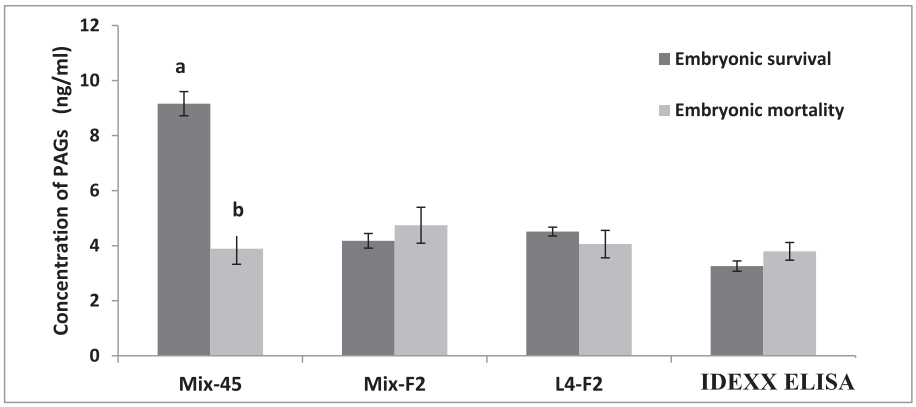

Fig. 1. Serum concentrations of bovine PAGs (mean ± SEM) in lactating dairy cows on day 30 (n = 183) of gestation for cows that either maintained pregnancy to day 60 of gestation (Embryonic survival; n = 141) or did not maintain pregnancy to day 60 of gestation (Embryonic mortality; n = 43) following FTAI. Each cow had a viable embryo based on presence of a heartbeat on day 30. Three antibody combinations (Mix-45, Mix-F2, and L4-F2) were utilized in an in-house ELISA or a commercial ELISA to detect PAGs in circulation. Only the Mix-45 antibody combination (positive control) detected a difference in serum concentrations of PAGs between cows that experienced embryonic survival or mortality (Mix-45; $^{ab}P < .001$).

mortality (AUC = 0.68; P < .0001; data not shown). Serum concentrations of PAGs detected with Mix-45 above 28.69 was 95% accurate in predicting the pregnancy maintenance through to parturition, whereas a concentration of PAG below 1.62 ng/ml was 95% accurate in predicting embryonic mortality between day 30 following FTAI to parturition (data not shown).

3.2. Experiment 2

Following FTET there were 131 cows confirmed pregnant via ultrasonography on day 30 (embryo transfer was performed on day 7). Pregnancy loss was determined between days 30–60, and there were 21 (16%) cows that experienced late embryonic mortality and 110 (84%) cows that maintained pregnancy until day 60 of gestation. A viable embryo, based on a heartbeat, was present on day 30 in all cows used for this experiment. Mean serum concentrations of PAGs, as detected with Mix-45 (P = .05) and the IDEXX PAG ELISA, were greater (P < .0001) in cows that maintained pregnancy compared with cows that experienced embryonic mortality at any point between days 30–60 of pregnancy (Fig. 4). However, there was no difference in maternal concentrations of PAGs on day 30 between cows that experienced embryonic survival or mortality when the Mix-F2 (P = .25) or L4-F2 (P = .42) antibody combinations were used. When circulating concentrations of PAGs on day 30 decreased (P < .05), the probability of diagnosing late embryonic mortality in cows following FTET increased when using the Mix-45 (P < .004) and the IDEXX PAG assays (P < .002) (Fig. 5), but not the other antibody combinations. Among the antibody combinations that were examined there was no difference in the area under the curve between the IDEXX PAG ELISA (AUC = 0.77) and Mix-45 (AUC = 0.76); the AUC with Mix-F2 was 0.63 and with L4/F2 was 0.6 in regards to detecting embryonic loss (Fig. 6, Table 2). Furthermore, utilizing the positive and negative predictive value analysis for the results from the Mix-45 assay, a circulating concentration of PAG >12.59 ng/ml was 95% accurate in predicting embryonic survival and a concentration of PAG below 1.85 ng/ml was 95% accurate in predicting embryonic mortality at day 30 of gestation (p < .0001). Utilizing the positive and negative predictive value analysis for the results from the IDEXX assay, a circulating concentration of PAG >4.72 ng/ml was 95% accurate in predicting embryonic survival and a concentration of PAG below 1.19 ng/ml was 95% accurate in predicting embryonic mortality at day 30 of pregnancy (P < .0001) after embryo transfer on day 7.

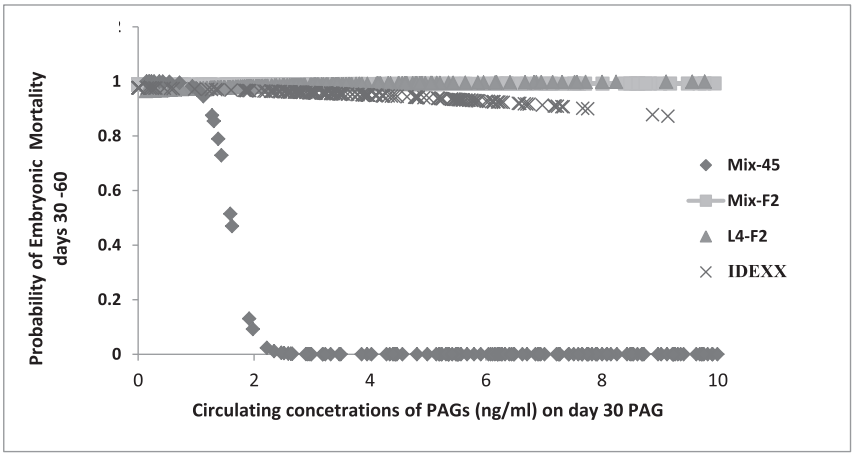

Fig. 2. The probability of pregnancy loss between days 30 and 60 of gestation following FTAI. Serum concentrations of PAGs on day 30 were detected by using four PAG assay platforms: Mix-45, Mix-F2, L4-F2, and a commercial (IDEXX) PAG ELISA. Decreased serum concentrations of PAGs as measured by the Mix-45 assay on day 30 significantly increased the probability of late embryonic mortality in lactating dairy cows following insemination (P < .01).

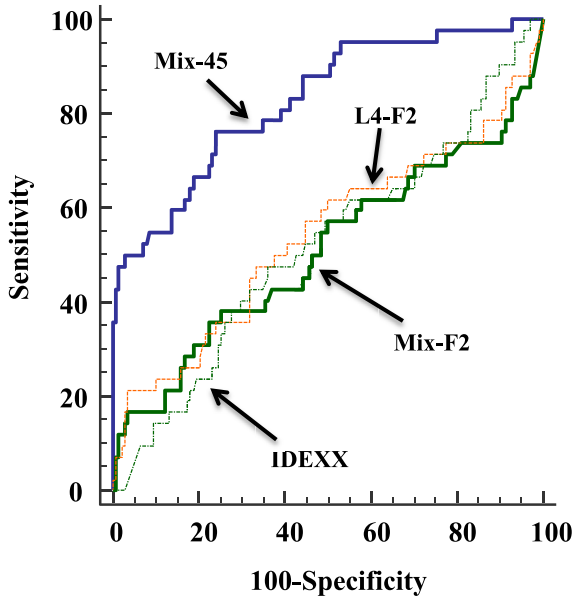

Fig. 3. Receiver operating characteristic (ROC; MedCal software) curves for three antibody combinations (Mix-45, Mix-F2, L4-F2) employed as in-house PAG ELISAs and a commercial PAG ELISA (IDEXX) were used to determine if circulating concentrations of PAGs on day 30 of gestation can predict late embryonic/fetal mortality in lactating dairy cows. Pregnancy loss occurred between days 30–60 following artificial insemination. The ROC curve graphically displays the relationship between true positive rate (Sensitivity) and false positive rate (100-Specificity) when an increasing cutoff for the PAG test was applied. When the true positive rate and the false positive rate both decrease as the cutoff value is increased this results in a diagonal line through the center meaning the test is not predictive (50:50 probability). However, when the line is deflected to the left of center the test is useful since it has a relatively high true positive rate and a low false positive rate at a specific cutoff. The accuracy of the diagnosis is explained by the area under the curve (AUC). The further the curve is shifted to the left the more accurate the prediction. Among the individual assays, the Mix-45 antibody combination (positive control) had the largest AUC (0.83; $P < .0001$), followed by L4-F2 (0.54), Mix-F2 (0.51) and a commercial ELISA from IDEXX (0.52).

4. Discussion

Circulating concentrations of PAGs on days 28–31 have been shown to be measurably lower in cows that experienced late embryonic mortality compared to animals that maintained a pregnancy [1–3,12,13,20,21]. However, such observations have not been observed in every study; for example, Ricci et al. reported that PAGs were not an accurate predictor of late embryonic mortality [15]. Since PAGs are members of a large gene family, we hypothesized that certain PAGs secreted during early gestation may be associated with embryonic mortality, whereas others may be less correlated with subsequent pregnancy loss. The use of different combinations of antibodies directed against PAGs produced during the first trimester may improve the predictability of pregnancy loss when incorporated into a PAG ELISA.

In cattle, a typical profile for circulating PAGs involves a rise between day 24 to day 32, followed by a transient plateau and decline by day 45 of gestation [3,10,15]; around day 60 circulating PAGs begin to increase steadily again until term when they rise dramatically and peak around parturition [8–10,22]. Importantly, the time just prior to day 24 in cattle is associated with adhesion of mononuclear cells of the trophectoderm to the uterine luminal epithelium and the first appearance of giant BNC (day 19–21) [23]. These cells arise in the trophectoderm of ruminants and migrate across the microvillar junction and fuse with nearby uterine epithelial cells [24,25]. After fusion of the bovine BNCs with the uterine epithelium, exocytosis of BNC secretory granules delivers their contents to the uterine stroma. Many bovine PAGs are packaged into these granules, which is why soon after exocytosis they begin to appear in the maternal circulation. From days 24–45 of gestation there is extensive tissue remodeling associated with bovine placentation [26]. Wooding et al. has speculated that certain PAGs expressed at the placenta-uterine interface may be associated with tissue turnover or remodeling due to the fact that some are functional proteinases [6,22,27]. Consequently, reduced circulating concentrations of PAGs on days 28–30 may reflect inadequate trophoblast development and (or) function and be predictive of embryonic mortality in cattle.

The discrepancy in the use of circulating PAG concentrations to predict pregnancy loss might be explained by the specific PAGs being detected by antibodies employed in the PAG ELISA. The reason for the differences in measurable concentrations of PAGs on day 30 is not known, but it may reflect varying affinities of the antibodies for different members of the PAG family. The monoclonal antibodies employed in this study (A6, J2, and L4) have been shown to detect multiple members of the PAG family present in cotyledonary extracts [10]: A6 antibody bound most prominently to PAG 4, 6, 7, and 16; J2 antibody bound PAG 20; and L4 antibody bound PAG 4, 6, 9, and 21. The preceding antibodies likely have a weak affinity for other members of the PAG family as well. Indeed, the capacity to bind to a range of differentially abundant PAGs during early gestation may prove to be a critical basis for predicting pregnancy loss between days 30 and 60 of gestation in cattle. Selective binding of the displayed PAGs in the ELISA by the different polyclonal antibodies used in these assays further broadens their capacity to detect differentially abundant PAG forms. Each of the different polyclonal antibodies was raised against purified PAGs

Table 1
Positive and negative predictive value analysis of circulating concentrations of PAGs from pregnancies established by FTAI.[a]

Abs	N[b]	Embryo Loss[c]	Embryo Maintenance[d]	AUC[e]	SEM	95% CI[f]	*P value*	+ Predictive Value[g]	- Predictive Value[h]
Mix-45	183	42 (23%)	141 (77%)	0.83	0.04	0.77 to 0.88	<.0001	≤1.6 ng/mL	≥8.4
Mix/F2	183	42 (23%)	141 (77%)	0.51	0.06	0.43 to 0.58	.89	NA	NA
L4/F2	183	42 (23%)	141 (77%)	0.54	0.06	0.47 to 0.62	.46	NA	NA
IDEXX	180	42 (23%)	138 (77%)	0.52	0.05	0.44 to 0.59	.71	NA	NA

[a] PAGs were detected in serum collected on day 30 following fixed-time artificial insemination.
[b] N = sample size.
[c] Number and percentage of cows that lost a pregnancy between days 31 and 60 after FTAI.
[d] Number and percentage of cows that maintained pregnancy to day 60.
[e] AUC – area under curve.
[f] CI- confidence interval for area under the curve.
[g] Cut-off level refers to the concentration of PAGs in serum below which (+ predictive value) or above which (- predictive value) embryonic mortality or survival was predicted to occur with 95% accuracy, respectively.
[h] Cut-off level refers to the concentration of PAGs in serum below which (+ predictive value) or above which (- predictive value) embryonic mortality or survival was predicted to occur with 95% accuracy, respectively.

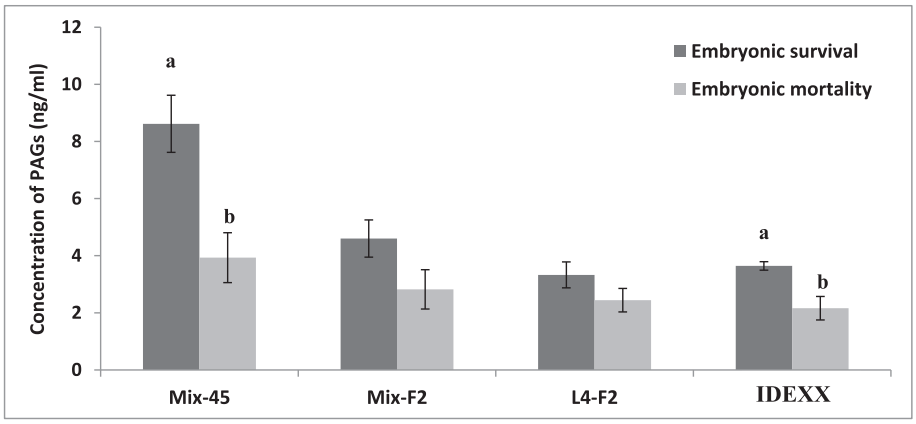

Fig. 4. The relationship between serum concentrations of bovine pregnancy associated glycoproteins (PAGs; mean ± SEM) in lactating dairy cows on day 30 (n = 131) of pregnancy for cows that either maintained pregnancy to day 60 of gestation (Embryonic survival; n = 110) or did not maintain pregnancy to day 60 of pregnancy (mortality; n = 21) following fixed-time ET. Each cow had a viable embryo based on presence of a heartbeat on day 30. Three antibody combinations (Mix-45, Mix-F2, and L4/F2) were utilized in an in-house ELISA or a commercial PAG ELISA to detect PAGs in circulation. The Mix-45 and the commercial PAG ELISA antibody combination detected a difference in serum concentrations of PAGs between cows that experienced embryonic survival or mortality. Cows that experienced late embryonic mortality had decreased serum concentrations of PAG on d 30 compared with cows that maintained pregnancy (Mix-F2, Commercial PAG ELISA; $^{ab}P<.0001$, respectively).

that differed somewhat in their composition (listed in the materials and methods section). Because of the range of PAGs that can be bound by the monoclonals and the polyclonal antibodies employed in these assays, it is not known definitely which specific members of the PAG family in the maternal circulation are recognized by these ELISAs. An added complexity resides in the fact that different members of the PAG family are expressed at different times throughout pregnancy [17,18,28] and expression of PAG transcripts may be dependent on the stage of gestation. For example, PAG-1 is not highly expressed early in gestation, whereas; PAG-4, -5, and -9 are detectable as early as day 25 after insemination [17,28].

In the current study, circulating concentrations of PAGs on day 30 were higher in cows that maintained pregnancy (9.16 ± 0.44 ng/ml) compared to cows that experienced pregnancy loss (3.89 ± 0.57 ng/ml) between days 30–60 of gestation with the Mix-45 ELISA (Fig. 1). Similar results have been reported in dairy cows [12,20]; however, this was not the case in other studies [15]. In an effort to more accurately monitor pregnancy loss, we assessed other PAG ELISAs alongside the Mix-45 antibody combination. In the first experiment, there was no difference in circulating concentrations of PAGs on day 30 following AI between cows that experienced embryonic/fetal survival or loss when the Mix-F2, L4-F2, or IDEXX PAG ELISAs were used. These data suggest that there are particular PAGs or PAG isoforms that are important for prediction of embryonic survival in cattle. The reason that the Mix-F2 and L4-F2, or IDEXX PAG ELISAs did not detect future pregnancy losses might be due to the relative reactivity of the antisera with the particular PAGs that were trapped by the monoclonal antibodies. In order to understand this phenomenon, PAGs present in the maternal circulation around day 30 must be characterized in more detail. There are more than 20 PAGs transcribed and expressed in the bovine placenta [16,22,28]. The half – life of PAGs seems to differ depending on when during gestation they are measured [3]. The source of the antibodies directed against PAGs employed in an ELISA may explain why different studies have shown different circulating concentrations and half-lives of PAGs. The antibodies used in this study were generated against different PAG isolates (differing in the gestational age of the extracted placentas as well as

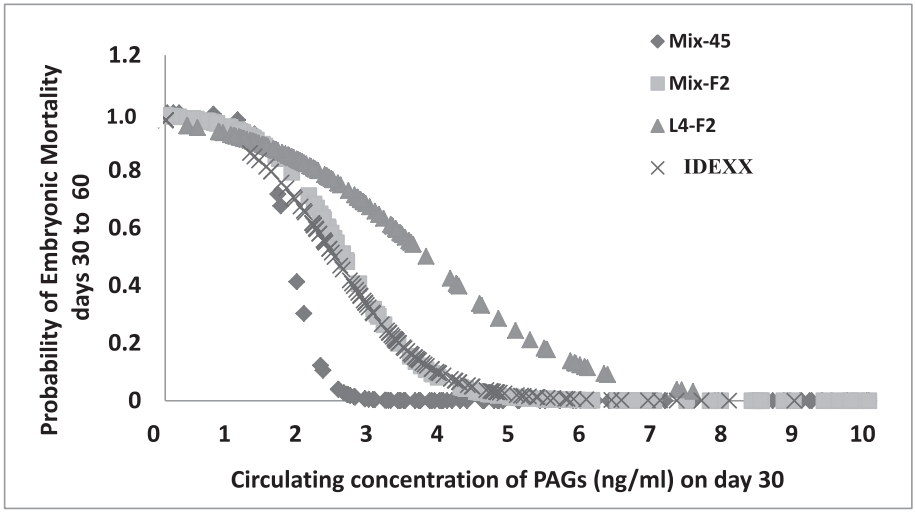

Fig. 5. Probability of late embryonic/fetal mortality following fixed-time ET between days 30–60 of gestation based on day 30 serum concentrations of PAGs (n = 131). Decreased serum concentrations of PAGs detected with Mix-45 (P < .004); the commercial PAG ELISA (P < .002) on day 30 significantly increased (P < .05) the probability of late embryonic mortality in lactating dairy cows following fixed-time ET.

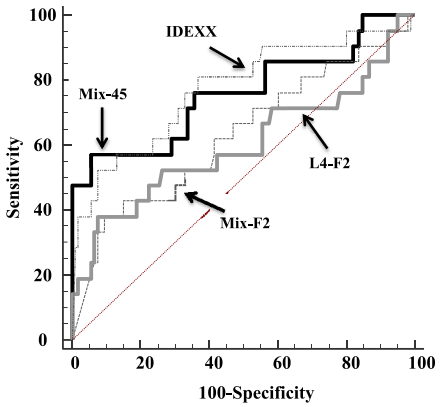

Fig. 6. Receiver operating characteristic (ROC; MedCal software) curves for PAG ELISAs comprised from three antibody combinations (Mix-45, Mix-F2, L4-F2) and a PAG ELISA from IDEXX was used to test the model that circulating concentrations of PAGs on day 30 of gestation can predict late embryonic mortality in lactating dairy cows. ROC curves were established by plotting the correlation between the specificity and sensitivity at numerous cut-off levels. Pregnancy loss occurred between days 30–60 following fixed-time embryo transfer. The accuracy of the diagnosis is explained by the area under the cure (AUC). The further the curve is shifted to the left the more accurate the prediction. Among the individual assays, Mix-45 assay and the commercial PAG ELISA was similar in the AUC 0.76, 0.77, respectively, whereas; the AUC with Mix-F2 and L4-F2 was 0.63 and 0.6, respectively.

in the purification schemes that were employed). The reactivity differences may also be due to antibody binding to differentially glycosylated PAG isoforms [29–31]. Enzymatic modification of proteins or glycosylation can affect protein characteristics such as their ability to bind with its receptor, and resistance to enzymatically degradation [32]. Glycosylation is a contributing factor that regulates the half-life of proteins in circulation. For instance, lutropin contains sulfated GaLNAc that is responsible for regulating lutropin clearance from circulation [33]. The asialoglycoprotein receptor binds to glycans with a terminal GaLNAc [34], which is responsible for the clearance of asialoglycoprotein from the circulation. Klisch et al. [30] suggested that the longer half-life of PAGs and higher circulating concentrations of PAGs during the peripartum period [8–10] might be explained by the loss of terminal GaLNAc. However, a demonstration that individual PAGs exhibit different clearance times has not been reported; even so, modulation of circulating half-life by post translational modifications seems plausible.

Additional statistical analyses were performed to evaluate circulating PAGs on day 30 as a way to predict pregnancy loss between days 30–60 of gestation. A ROC curve was predictive for the Mix-45 assay but not for other antibody combinations (Fig. 3). The lower PAG concentrations in cows that lost pregnancy may reflect a decreased number of binucleate giant cells and (or) a compromised placenta. Somatic cell nuclear transfer (SCNT) usually results in an increase in embryonic mortality in cattle. The cause of such pregnancy loss is thought to be related to abnormal placental development as well as the number of placentomes [35]. Some of the mRNAs encoding for PAGs were decreased in SCNT pregnancies [36]. Despite the lower PAG transcript abundance in SCNT pregnancies, circulating PAG concentrations were often not lower in recipient cows receiving SCNT-derived embryos [37,38]. These counterintuitive observations are difficult to reconcile, as are many aspects of SCNT pregnancies.

In an effort to more accurately monitor embryonic loss following FTET, experiment 2 involved measuring circulating concentrations of PAGs 23 days after FTET (embryo transfer was performed on day 7) as a marker for predicting pregnancy loss between days 30–60 of pregnancy. Mean serum concentrations of PAGs, as detected with Mix-45 and IDEXX ELISAs, were greater in cows that maintained pregnancy compared with cows that subsequently experienced pregnancy loss between days 30–60 of gestation (Fig. 4). An additional analysis with a ROC curve was predictive for the Mix-45 assay and the IDEXX PAG assay (Fig. 6). Currently it is not clear why the IDEXX PAG ELISA was predictive for detecting pregnancy loss following FTET, but not FTAI. Circulating concentrations of PAGs may increase more rapidly following FTET compared to FTAI. Alternatively, in vitro produced embryos may express different members of the PAG family compared to in vivo produced embryos. In a previous study, embryo quality or stage of embryonic development at embryo transfer did not result in differences in circulating concentrations of PAGs on day 28 of gestation [3]. Consequently, the basis for the observed differences in measurable PAG (and their predictive capacity) between FTET and FTAI are unclear at this time.

5. Conclusion

In summary, the bovine PAG family is large (>20 members) and expression of PAG members varies with stage of gestation [18,22]. The results in this manuscript demonstrate that different antibody combinations are capable of recognizing different PAGs or differing amounts of PAG isoforms that are associated with viability of the pregnancy. The measurable differences among PAG ELISAs in predicting losses between day 30 and 60 of gestation may reflect varying affinities of different antibodies for different members of the PAG family. Other research groups have also described how different PAG antisera appear to recognize distinct PAG populations

Table 2
Positive and negative predictive value analysis of circulating concentrations of PAGs from pregnancies established by FTET.[a]

Abs	N[b]	Embryo Loss[c]	Embryo Maintenance[d]	AUC[e]	STM	95% CI[f]	*P value*	+ Predictive Value[g]	- Predictive Value[h]
Mix/45	131	21 (16%)	110 (84%)	0.76	0.07	0.67 to 0.82	.0001	≤1.8	≥12.6
Mix/F2	131	21 (16%)	110 (84%)	0.63	0.07	0.53 to 0.71	.08	NA	NA
L4/F2	131	21 (16%)	110 (84%)	0.6	0.08	0.51 to 0.68	.2	NA	NA
IDEXX	127	21 (16.5%)	106 (83%)	0.77	0.06	0.68 to 0.84	<.0001	≤1.2	≥4.7

[a] PAGs were detected in serum collected on day 30 after estrus and following fixed-time embryo transfer.
[b] N = sample size.
[c] Embryonic mortality or fetal loss between days 31–60 representing the number of cows that had lost pregnancy and the percentage of embryonic loss.
[d] Embryonic survival represent the number (%) of cows that maintained pregnancy from days 31–60 of gestation.
[e] AUC – area under curve.
[f] CI-confidence interval based on the area under the curve.
[g] Cut-off level refers to the concentration of PAGs in serum below which (+ predictive value) or above which (- predictive value) embryonic mortality or survival is predicted to occur with 95% accuracy, respectively.
[h] Cut-off level refers to the concentration of PAGs in serum below which (+ predictive value) or above which (- predictive value) embryonic mortality or survival is predicted to occur with 95% accuracy, respectively.

[39–41]. Most importantly, the data presented in this manuscript suggest that distinct PAG isoforms, or different concentrations of circulating PAGs, are correlated with placental function. This work demonstrated that it is possible to measure some of these biologically meaningful correlations with certain antibody combinations. Currently, we do not know definitely which specific PAGs in the maternal circulation are selectively bound by the antibodies employed in the assays. As more specific reagents become available (e.g. PAG-specific monoclonal antibodies), we will be able to more fully understand how individual PAG forms are correlated with pregnancy status in cattle and other ruminants.

Declaration of interest

JAG is listed as an inventor on patents related to the detection of PAGs in pregnant females.

Acknowledgements

The authors would like to acknowledge the University of Missouri Research Board for financial support.

References

[1] Pohler KG, Pereira MHC, Lopes FR, Lawrence JC, Keisler DH, Smith MF, et al. Circulating concentrations of bovine pregnancy-associated glycoproteins and late embryonic mortality in lactating dairy herds. J Dairy Sci 2016;99:1584–94.
[2] Pohler KG, Peres RFG, Green JA, Graff H, Martins T, Vasconcelos JLM, et al. Use of bovine pregnancy-associated glycoproteins to predict late embryonic mortality in postpartum Nelore beef cows. Theriogenology 2016;85:1652–9.
[3] Pohler KG, Geary TW, Johnson CL, Atkins JA, Jinks EM, Busch DC, et al. Circulating bovine pregnancy associated glycoproteins are associated with late embryonic/fetal survival but not ovulatory follicle size in suckled beef cows. J Anim Sci 2013;91:4158–67.
[4] Santos JEP, Bartolome JA, Cerri RLA, Juchem SO, Hernandez O, Trigg T, et al. Effect of a deslorelin implant in a timed artificial insemination protocol on follicle development, luteal function and reproductive performance of lactating dairy cows. Theriogenology 2004;61:421–35.
[5] Diskin MG, Morris DG. Embryonic and Early Foetal Losses in cattle and other ruminants. Reprod Domest Anim 2008;43:260–7.
[6] Wooding FBP, Roberts RM, Green JA. Light and electron microscope immunocytochemical studies of the distribution of Pregnancy-Associated Glycoproteins (PAGs) throughout pregnancy in the cow: possible functional implications. Placenta 2005;26:807–27.
[7] Zoli AP, Demez P, Beckers JF, Reznik M, Beckers A. Light and electron microscopic immunolocalization of bovine pregnancy- associated glycoprotein in the bovine placentome. Biol Reprod 1992;46:623–9.
[8] Sasser RG, Ruder CA, Ivani KA, Butler JE, Hamilton WC. Detection of pregnancy by radioimmunoassay of a novel pregnancy- specific protein in serum of cows and a profile of serum concentrations during gestation. Biol Reprod 1986;35:936–42.
[9] Zoli AP, Guilbault LA, Delahaut P, Ortiz WB, Beckers JF. Radioimmunoassay of a bovine pregnancy-associated glycoprotein in serum: its application for pregnancy diagnosis. Biol Reprod 1992;46:83–92.
[10] Green JA, Parks TE, Avalle MP, Telugu BP, McLain AL, Peterson AJ, et al. The establishment of an ELISA for the detection of pregnancy-associated glycoproteins (PAGs) in the serum of pregnant cows and heifers. Theriogenology 2005;63:1481–503.
[11] Perry GA, Smith MF, Lucy MC, Green JA, Parks TE, MacNeil MD, et al. Relationship between follicle size at insemination and pregnancy success. Proceedings of the National Academy of Sciences of the United States of America 2005;102:5268–73.
[12] Breukelman SP, Perényi Z, Taverne MAM, Jonker H, van der Weijden GC, Vos PLAM, et al. Characterisation of pregnancy losses after embryo transfer by measuring plasma progesterone and bovine pregnancy-associated glycoprotein-1 concentrations. Vet J 2012;194:71–6.
[13] Giordano JO, Guenther JN, Lopes G, Fricke PM. Changes in serum pregnancy-associated glycoprotein, pregnancy-specific protein B, and progesterone concentrations before and after induction of pregnancy loss in lactating dairy cows. J Dairy Sci 2012;95:683–97.
[14] Humblot P. Use of pregnancy specific proteins and progesterone assays to monitor pregnancy and determine the timing, frequencies and sources of embryonic mortality in ruminants. Theriogenology 2001;56:1417–33.
[15] Ricci A, Carvalho PD, Amundson MC, Fourdraine RH, Vincenti L, Fricke PM. Factors associated with pregnancy-associated glycoprotein (PAG) levels in plasma and milk of Holstein cows during early pregnancy and their effect on the accuracy of pregnancy diagnosis. J Dairy Sci 2015;98:2502–14.
[16] Telugu BP, Walker A, Green J. Characterization of the bovine pregnancy-associated glycoprotein gene family - analysis of gene sequences, regulatory regions within the promoter and expression of selected genes. BMC Genom 2009;10:185.
[17] Patel OV, Yamada O, Kizaki K, Takahashi T, Imai K, Hashizume K. Quantitative analysis throughout pregnancy of placentomal and interplacentomal expression of pregnancy-associated glycoproteins-1 and -9 in the cow. Mol Reprod Dev 2004;67:257–63.
[18] Touzard E, Reinaud P, Dubois O, Guyader-Joly C, Humblot P, Ponsart C, et al. Specific expression patterns and cell distribution of ancient and modern PAG in bovine placenta during pregnancy. Reproduction 2013;146:347–62.
[19] Wildman EE, Jones GM, Wagner PE, Boman RL, Troutt HF, Lesch TN. A dairy cow body condition scoring system and its relationship to selected production characteristics. J Dairy Sci 1982;65:495–501.
[20] Thompson IM, Cerri RLA, Kim IH, Green JA, Santos JEP, Thatcher WW. Effects of resynchronization programs on pregnancy per artificial insemination, progesterone, and pregnancy-associated glycoproteins in plasma of lactating dairy cows. J Dairy Sci 2010;93:4006–18.
[21] Szenci O, Beckers JF, Sulon J, Bevers MM, Börzsönyi L, Fodor L, et al. Effect of induction of late embryonic mortality on plasma profiles of pregnancy associated glycoprotein 1 in heifers. Vet J 2003;165:307–13.
[22] Wallace RM, Pohler KG, Smith MF, Green JA. Placental PAGs: gene origins, expression patterns, and use as markers of pregnancy. Reproduction 2015;149:R115–26.
[23] Wooding FBP. Frequency and localization of binucleate cells in the placentomes of ruminants. Placenta 1983;4:527–40.
[24] Wooding FBP. Structure and function of placental binucleate (giant) cells. Bibl Anat 1982;22:134–9.
[25] Wooding FBP. The role of binucleate cell in ruminant placental structure. Reprod Fertil 1982;31:31–9.
[26] Assis Neto AC, Pereira FTV, Santos TC, Ambrosio CE, Leiser R, Miglino MA. Morpho-physical recording of bovine conceptus (Bos indicus) and placenta from days 20 to 70 of pregnancy. Reprod Domest Anim 2010;45:760–72.
[27] Telugu BPVL, Palmier MO, Van Doren SR, Green JA. An examination of the proteolytic activity for bovine pregnancy-associated glycoproteins 2 and 12. Biol Chem 2010;391:259–70.
[28] Green JA, Xie S, Quan X, Bao B, Gan X, Mathialagan N, et al. Pregnancy-associated bovine and ovine glycoproteins exhibit spatially and temporally distinct expression patterns during pregnancy. Biol Reprod 2000;62:1624–31.
[29] Klisch K, Jeanrond E, Pang P-C, Pich A, Schuler G, Dantzer V, et al. A tetraantennary glycan with bisecting N-Acetylglucosamine and the sda antigen is the predominant N-Glycan on bovine pregnancy-associated glycoproteins. Glycobiology 2008;18:42–52.
[30] Klisch K, Boos A, Friedrich M, Herzog K, Feldmann M, Sousa N, et al. The glycosylation of pregnancy-associated glycoproteins and prolactin-related protein-I in bovine binucleate trophoblast giant cells changes before parturition. Reproduction 2006;132:791–8.
[31] Klisch K, Leiser R. In bovine binucleate trophoblast giant cells, pregnancy-associated glycoproteins and placental prolactin-related protein-I are conjugated to asparagine-linked N-acetylgalactosaminyl glycans. Histochem Cell Biol 2003;119:211–7.
[32] Sinclair AM, Elliott S. Glycoengineering: the effect of glycosylation on the properties of therapeutic proteins. J Pharmaceut Sci 2005;94:1626–35.
[33] Mi Y, Shapiro SD, Baenziger JU. Regulation of lutropin circulatory half-life by the mannose/N-acetylgalactosamine-4-SO4 receptor is critical for implantation in vivo. J Clin Invest 2002;109:269–76.
[34] Coombs PJ, Taylor ME, Drickamer K. Two categories of mammalian galactose-binding receptors distinguished by glycan array profiling. Glycobiology 2006;16:1C–7C.
[35] Lee RSF, Peterson AJ, Donnison MJ, Ravelich S, Ledgard AM, Li N, et al. Cloned cattle fetuses with the same nuclear genetics are more variable than contemporary half-siblings resulting from artificial insemination and exhibit fetal and placental growth deregulation even in the first trimester. Biol Reprod 2004;70:1–11.
[36] Hashizume K, Ishiwata H, Kizaki K, Yamada O, Takahashi T, Imai K, et al. Implantation and placental development in somatic cell clone recipient cows. Clon Stem Cell 2002;4:197–209.
[37] Constant F, Camous S, Chavatte-Palmer P, Heyman Y, de Sousa N, Richard C, et al. Altered secretion of pregnancy-associated glycoproteins during gestation in bovine somatic clones. Theriogenology 2011;76:1006–21.
[38] Chavatte-Palmer P, de Sousa N, Laigre P, Camous S, Ponter AA, Beckers JF, et al. Ultrasound fetal measurements and pregnancy associated glycoprotein secretion in early pregnancy in cattle recipients carrying somatic clones. Theriogenology 2006;66:829–40.
[39] Lopez-Gatius F, Hunter RHF, Garbayo JM, Santolaria P, Yaniz J, Serrano B, et al. Plasma concentrations of pregnancy-associated glycoprotein-1 (PAG-1) in high producing dairy cows suffering early fetal loss during the warm season. Theriogenology 2007;67:1324–30.
[40] Perényi ZS, Szenci O, Sulon J, Drion PV, Beckers JF. Comparison of the ability of three radioimmunoassay to detect pregnancy-associated glycoproteins in bovine plasma. Reprod Domest Anim 2002;37:100–4.
[41] Reese ST, Pereira MHC, Edwards JL, Vasconcelos JLM, Pohler KG. Pregnancy diagnosis in cattle using pregnancy associated glycoprotein concentration in circulation at day 24 of gestation. Theriogenology. 106:178–185.