Animal Reproduction Science 205 (2019) 115–125

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Temporal changes in plasma profile of pregnancy-associated glycoprotein, progesterone and estrone sulfate associated with fetal number during early- and mid-pregnancy in goats

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ARTICLE INFO

Keywords:
Pregnancy-associated glycoprotein
Progesterone
Estrone sulfate
Plasma
Pregnancy
Goat

ABSTRACT

This study was designed to investigate plasma profile of pregnancy-associated glycoprotein (PAG), progesterone (P4) and estrone sulfate (E_1S) during early- and mid-pregnancy. The goal was to explore the relationships with values for reproductive variables, to detect the most reliable predictor variable, and to identify the most desirable time point for blood collection for determining fetal number in goats. After ultrasonographic examination at d35–40 post-mating, blood sampling of 15 pregnant goats (total 18) was continued until d114. The PAG profile was characterized by gradual increase during early pregnancy from d26 to d51 and thereafter concentrations were relatively constant until d114 of gestation. The effect of fetal number on plasma PAG, P4 and E_1S was first evident on d28, d51 and d26, respectively. During mid-pregnancy, does with twins had a greater ($P < 0.05$) PAG (S-N = 2.54 ± 0.12 compared with 1.59 ± 0.11), P4 (18.91 ± 0.67 compared with 14.51 ± 0.47 ng/mL) and E_1S (16.34 ± 0.76 compared with 11.32 ± 0.44 ng/dL) as compared with does with a singleton fetus. Plasma PAG but not P4 and E_1S was positively correlated with fetal number and birth weight of kids during early pregnancy. Multivariate linear regression and discriminant function analyzes allowed for identification of plasma PAG as the most reliable predictor for fetal number and birth weight of kids. Furthermore, d58 was the most suitable single time point for prediction of fetal number using PAG as a biomarker. In conclusion, plasma profile of PAG, P4 and E_1S was affected by fetal count. Plasma PAG was identified as the most reliable predictor variable of fetal number and birth weight of kids as compared to plasma P4 and E_1S.

1. Introduction

In farm animals, including goats, the ovarian and feto-placental unit functions as endocrine and paracrine organ by secreting a wide range of chemical messengers into the maternal circulation (Hoffmann and Schuler, 2002). Hormones such as the pregnancy-associated glycoproteins (PAG), progesterone (P4) and estrone sulfate (E_1S) are valuable markers for pregnancy determination and fetal well-being in goats.

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https://doi.org/10.1016/j.anireprosci.2019.04.010
Received 27 January 2019; Received in revised form 5 March 2019; Accepted 22 April 2019
Available online 24 April 2019
0378-4320/

Pregnancy-associated glycoproteins, a family of inactive aspartic proteinases, are abundantly present and released into the maternal circulation during gestation from the trophoblastic placental layer (Wooding et al., 2005). Thus, the presence of a measurable amount of PAGs in peripheral circulation can provide a basis to confirm pregnancy or to indicate the feto-placental functional status in goats.

Similarly, an adequate supply of P4 is essential for implantation, embryonic development and maintenance of pregnancy (Mann and Lamming, 1999). Monitoring of pregnancy in goats only on the basis of circulating P4, however, can lead to inaccurate determinations because concentrations may be associated with certain physio-pathological conditions such as the presence of luteal cysts, late return to estrus and pseudopregnancy (Charallah et al., 2010). A sulfo-conjugated estrogen, E_1S, is the major placental estrogen in maternal circulation of ruminants during pregnancy (Echternkamp, 1993) and can be detected in the peripheral plasma during gestation. Concentration of E_1S has also been used for detection of pregnancy and monitoring of fetal development in farm animals (Hirako et al., 2002).

The knowledge with regard to the profile of PAG, P4 and E_1S during early- and mid-pregnancy and associations with dam-related reproductive variables such as the fetal number, gestation length and birth weight of kids could be helpful for accurate pregnancy diagnosis, monitoring of pregnancy, and appropriate management of pregnant does during late pregnancy. The relationship of P4 and E_1S with feto-placental function and for prediction of fetal wellbeing has been documented in cattle but there have been few attempts to predict fetal numbers by the estimation of plasma PAG (de Sausa et al., 1999) or P4 and E_1S (Haldar et al., 2013) in goat. Furthermore, there is inadequate knowledge about associations of changes in different pregnancy hormone concentrations and with other reproductive variables, during early- and mid-pregnancy in goats. For such estimations, serial blood sampling is required. An identification of the most important single time point during pregnancy when concentrations of these hormones could be the most reliable predictor variable for determining important aspects of reproductive status will help to reduce time devoted to and cost involved in making such predictions and assist in management of pregnant animals.

The hypothesis was tested that circulating concentrations of specific pregnancy hormones (PAG, P4 and E_1S) is associated with number of fetuses and other reproductive variables during early- and mid-pregnancy in goats. The present study was conducted, therefore, with the objectives to (1) characterize the peripheral plasma concentrations of PAG, P4 and E_1S in does with single or twin fetuses during early- and mid-gestation, (2) investigate the relationship of plasma concentrations of these pregnancy hormones with reproductive variables such as fetal number, gestation length and birth weight of kids and (3) determine the most reliable predictor variable and a single blood sampling time point to discriminate singleton or twin bearing goat does.

2. Materials and methods

2.1. Experimental animals and their management

Eighteen apparently healthy and clinically diseases free multiparous (3–4 parity; weighing 35.4 ± 1.7 kg) estrous cyclic, native goats from the North India were included in the study. The animals were maintained as part of the flock of ICAR-Central Institute for Research on Goats, Makhdoom, Mathura, India (169 m above mean sea level; latitude 10∘N and longitude 78∘02′ E). The animals were group housed and reared in a semi-intensive system of management with uniform nutritional conditions and free access to water throughout the study. Goats were bred 10–12 h after onset of a natural estrus using fertility proven bucks. All the experimental procedures were conducted in accordance with good veterinary practices and approved by the Animal Ethics Committee of the Institute.

2.2. Blood sampling and transrectal ultrasonography

Blood samples were collected at different time points from d 7 until the end of the first month (i.e. < d 7, 10, 14, 18, 20, 22, 26, 28 and 30) followed by blood sampling at a weekly interval during rest of the experiment (from > d 30 until d 114). Approximately 5 mL blood samples were collected via jugular venipuncture into 10 mL plasma dipotassium (K2)-EDTA evacuated tubes (Vacutainer, BD, Franklin Lake, NJ) and immediately placed into the ice box. After centrifugation at 2500 × *g*, 10 min at 4 °C, plasma was harvested and stored at -20 °C.

On d 35–40 after mating, transrectal ultrasonography was performed in all the animals using an ultrasonic device (Just Vision 200-Model SSA- 320 A, Diagnostic Ultrasonography System, Toshiba, Japan) equipped with a real time convex array trans rectal transducer (PVF-738 F) of variable frequency (5–7 MHz). When conducting ultrasonography assessment of the pregnant uterus, there was imaging of an anechoic embryonic vesicle (black) surrounding the echoic (white) elongated streak (fetus) extending through more than half of fetal fluid. Ultrasonographic examination revealed that of 18 goats, 15 does were pregnant and three were non-pregnant. Non-pregnant animals were excluded from the study. Thus, the numbers of single- and twin-pregnancies were 7 and 8, respectively. The animals were grouped accordingly and blood samples were assayed.

2.3. Hormone assays

2.3.1. Assay for pregnancy-associated glycoprotein (PAG)

The concentrations of PAG in plasma samples were determined using a commercially available antigen capture enzyme-linked immunosorbent assay (ELISA; IDEXX Laboratories, Westbrook, ME, USA) according to the manufacturer's instructions. Briefly, 100 µL of plasma samples and assay controls (both positive and negative) were pipetted into 96-well, antiPAG antibody coated plates along

with 25 μL of sample diluent, sealed, and incubated for 60 min at 37 °C. After incubation and four washings, wells were incubated with detector solution (100 μL; anti-PAG antibody) for 30 min. Thereafter, plates were washed (4 ×) using a plate washer (Plate washer Hydroflex, Tecan) before addition of 100 μL of conjugate solution (anti-IgG-horseradish peroxidase) and there was incubation for 30 min at room temperature (RT). Following four serial washings, 100 μL of TMB substrate solution was added and incubated for 15 min at RT. Reactions were subsequently stopped by adding 100 μL of stop solution and absorbance was determined at 450 nm and at 630 nm (Tecan Sun Rise with Magellan 4.0 Software, Austria). The intra- and inter-plate coefficients of variations (CVs) for positive controls were 5.7% and 10.5%, respectively.

Results were calculated for each sample by subtraction of corrected mean sample absorbance with corrected mean absorbance of negative controls ($n = 4$; corrected absorbance = absorbance at 450 nm–absorbance at 630 nm) and expressed as the sample–negative (S-N). Pregnancy outcomes were determined based on the cut-off value (S-N = 0.3) as determined by the PAG ELISA manufacturer. For the results (S-N) ≥ 0.3, samples were classified as positive (pregnant), and those < 0.3 were classified as negative (non-pregnant).

2.3.2. Assay for progesterone (P4)

Quantitative determination of P4 concentration in plasma samples was conducted in duplicate using an ELISA kit following the manufacturer's instruction (DRG Diagnostics, Germany). It involved pipetting 25 μL of test references, controls and test samples into the antibody coated microtiter plate. After incubation for 5 min at RT, 200 μL of enzyme conjugate was added to all the wells and mixed gently for 1 min. The microtiter plate was covered and incubated for 1 h at 25 °C. After incubation, the contents of the microplate were decanted and three washings were performed (Plate washer Hydroflex, Tecan). Substrate solution (200 μL) was subsequently added to all wells and incubated for 15 min at room temperature before adding 50 μL of stop solution. The absorbance in each well was determined immediately using an ELISA plate reader at 450 nm (Tecan Sun Rise with Magellan 4.0 Software, Austria). The analytical sensitivity of the assay was 0.045 ng/ml. Intra- and inter-assay CVs for P4 were 6.1% and 8.6%, respectively. The range of reliable quantitation for the assay was 0–40 ng/mL. Before estimation of the P4 concentration in the goat plasma samples using a commercial kit, linearity-of-dilution was measured for estimation of accuracy of the ELISA assay and its compatibility with the sample matrix.

2.3.3. Assay for estrone sulphate (E_1S)

Plasma E_1S concentrations were quantified by using a commercially available goat E_1S ELISA kit (M/s Bioassay Technology Laboratory, Shanghai, China). The protocol used was that provided by the manufacturer. Briefly, frozen plasma samples were thawed at RT and vortexed before pipetting of samples (40 μL) or standards into the appropriate wells pre-coated with E_1S monoclonal antibody. Biotin conjugated anti-ES antibody (10 μL) was added to sample wells and 50 μL of enzyme conjugate (streptavidin-HRP) was subsequently dispensed into each sample and standard well. After incubation for 1 h at 37 °C, the microtiter plates were washed five times with diluted 1× wash buffer (300 μL per well) and blotted on paper towels to remove residual droplets. Subsequently, 50 μL of substrate solution A and B were added to each well. After 10 min of incubation in the dark at 37 °C, 50 μL of stop solution was added. The absorbance was quantified immediately at 450 nm with a plate reader. The intra- and inter-assay CVs were 7.8% and 9.7%, respectively.

2.4. Statistical analyzes

For statistical analyzes and presentation of results, the entire experiment was divided into early- (d 7 to d 51) and mid- (d 58 to d 114) pregnancy periods. The Linear Mixed Model procedure was used for type of pregnancy (single or twin fetus) as a dependent variable. 'Treatment' (type of pregnancy) and 'Time' (day after breeding) were included as fixed factors, sampling time as repeated effects, does as a random factor and the respective interactions were included in the model. The diagonal covariance structure followed by Bonferroni correction was used to estimate level of significance of treatment, time and the interaction for each dependent variable during early- and mid-pregnancy. For comparisons of PAG, P4 and ES concentrations among the groups at individual time point, an 'independent sample t-test' or corresponding non-parametric test (Mann–Whitney U test) was performed. Differences were considered significant at probability value (P) < 0.05 and a trend was defined at $P < 0.1$ and > 0.05. All data are presented as mean ± standard error of the mean.

Correlation coefficients between variables were calculated with Pearson correlation analysis. Data for reproductive variables obtained at the time of kidding such as number of fetuses, gestation length, birth wt. of kids and fetal sex, and plasma concentrations of pregnancy hormones (PAG, P4 and E_1S) were used. Stepwise multivariate linear regression analyses were conducted to identify significant independent predictors of the number of fetuses and birth weight of kids. The plasma PAG, P4 and ES concentrations (as independent variable) as well as number of fetuses or birth weight of kids (as dependent variables) were included into the models. Furthermore, a predictive model of group membership was developed based on the observed hormone concentrations of each case to recognize the most appropriate timing of blood sampling for predicting kidding size. This procedure generated a discriminant function using canonical discriminant function coefficients based on linear combinations of the predictor variables that provided the most precise discrimination between sampling time points. The stepwise discriminant function analysis used in this study has been described elsewhere (Haldar et al., 2013).

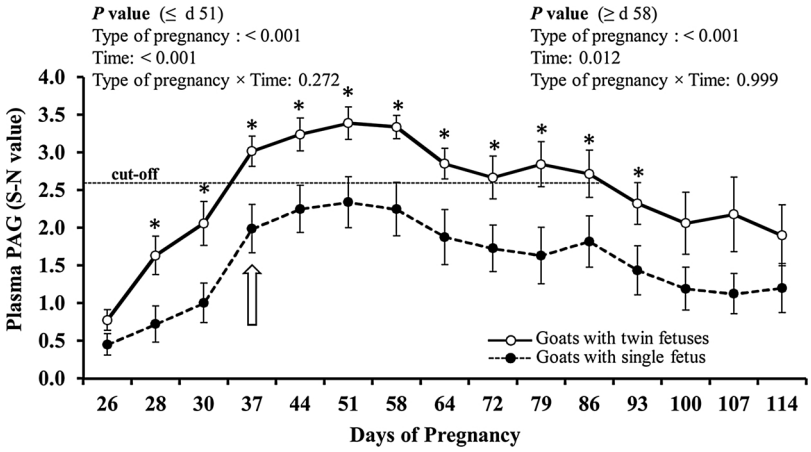

Fig. 1. Dynamic profiles of circulating concentrations of pregnancy-associated glycoprotein (PAG) (mean signal-noise; S-N ± SEM) quantified using an ELISA in singleton (dotted line and closed circle) or twin (solid line and open circle) fetus bearing goat does during early and mid-pregnancy; Early pregnancy represents gestation period from d 26 until d 51, whereas mid pregnancy is the duration from d 58 until d114 of gestation; Type of pregnancy indicates singleton or twin fetuses; The arrow and cut-off line indicates earliest time point and the S-N value to differentiate number of fetuses (from d 37 until d 86 of pregnancy), respectively; *$P < 0.05$.

3. Results

Pregnancy in the does was determined using ultrasonography, assessment of pregnancy hormones (PAG, P4 and E_1S) utilizing an ELISA, and ultimately kidding. Based on the results from these assessments, of 18 goats, seven had a single fetus, eight had twin fetuses and three were non-pregnant. Overall, the mean gestation period of does was 144.1 ± 1.6 days (138–150 days). The mean gestation lengths (days) for does with single and twin fetuses were 149.0 ± 1.04 (144–153) and 144.3 ± 0.84 (141–147), respectively. Gestation length was inversely associated with the fetal number ($r = -0.682$; $P < 0.001$) and birth weight of kids ($r = -0.372$; $P < 0.001$)

3.1. Plasma pregnancy-associated glycoprotein (PAG) profile

To determine the temporal profile of circulating PAG during early- and mid-pregnancy in goats, data from singleton and twin pregnancies were analyzed and there is depiction of these data in Fig. 1. The PAG profile was characterized by the gradual increase in plasma concentration during early pregnancy (from d 26 to d 51) with the greatest concentration being on d 51 of gestation. Thereafter, the concentration of PAG remained relatively consistent until the end of experiment (Fig. 1). Results from the mixed model analysis along with pairwise comparison indicated there was a difference ($P < 0.001$) between groups (single compared with twin birth) during the study period. The effect of number of fetuses on plasma PAG was first observed on d 28 of pregnancy and the differences in values for PAG remain consistent until d 93 ($P < 0.05$). Overall, mean plasma PAG concentration was 1.6 fold (1.4–2.6) greater in does with twin fetuses than to singleton bearing goats.

3.2. Plasma progesterone (P4) profile

Two samples with different P4 concentrations (11.31 and 24.96 ng/mL) were used for estimation for suitability of the ELISA system for the sample matrix. The means (± SEM) for plasma P4 profile in pregnant goats bearing different numbers of fetuses are depicted in Fig. 2. During pregnancy, the plasma P4 value in goats with single or twin fetuses fluctuated between 2.53 and 21.55 ng/mL, and 6.12 and 24.92 ng/ml, respectively. There was no effect of type of pregnancy (single or twin fetuses) on plasma P4 concentration during early pregnancy (d 7 to d 51 of gestation; Fig. 2). During mid-pregnancy, however, there was a greater ($P < 0.001$) P4 concentration in does with twin fetuses (18.91 ± 0.67 ng/mL) than does with a single fetus (14.51 ± 0.47 ng/mL). There was no interaction of values of kid size and day of sampling on plasma P4 concentration in the present study.

3.3. Plasma estrone sulfate (E_1S) profile

The temporal profile of plasma E_1S (mean ± SEM) in pregnant goats bearing single or twin fetuses is depicted in Fig. 3. During the first month of gestation (from d 7 to d 26) concentrations of E_1S in both the groups were similar (Fig. 3). Between d 30 and d 114 of pregnancy, mean plasma E_1S concentration was greater ($P < 0.001$) in does with twin fetuses than does bearing a single fetus. In goats with twin fetuses, plasma E_1S concentration started to increase from d 22 of gestation and the greatest concentration was at d 58 post-conception. Thereafter, concentrations of E_1S remained relatively constant until d 114 of pregnancy. The rate of increase in E_1S concentration from days 22–58 was 2.41 fold greater in ewes with twin fetuses than in those with a single fetus. Overall, the mean

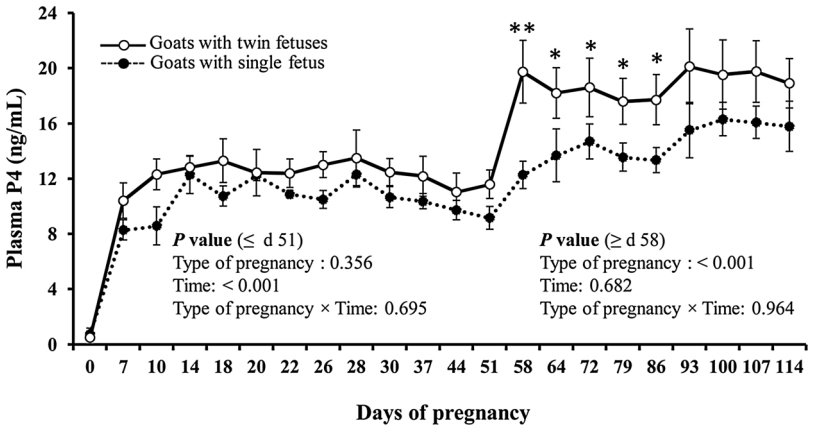

Fig. 2. Dynamic profiles of circulating progesterone (P4; mean ± SEM) quantified using an ELISA in singleton (dotted line and closed circle) or twin (solid line and open circle) fetus bearing goat does during early and mid-pregnancy; Early pregnancy represents gestation period from d 7 until d 51, whereas mid pregnancy is the duration from d 58 until d114 of gestation; Type of pregnancy indicates singleton or twin fetuses; *$P < 0.05$; **$P < 0.01$.

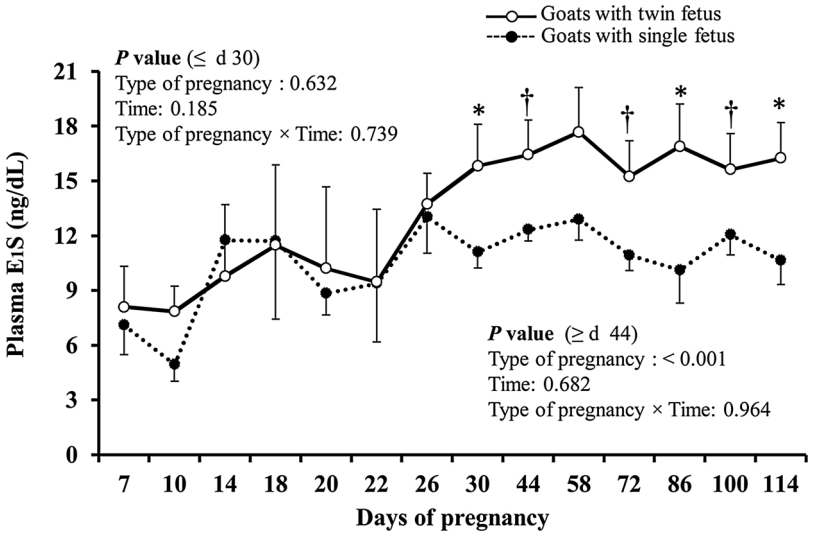

Fig. 3. Dynamic profiles of circulating estrone sulfate (E_1S) (mean ± SEM) quantified using an ELISA in singleton (dotted line and closed circle) or twin (solid line and closed square) fetus bearing pregnant goat does during early and mid-pregnancy; For mixed model analysis, entire period was divided in to pregnancy period 1 (from d 7 until d 26 of gestation) and pregnancy period 2 (from d 30 until d 114 of gestation); Type of pregnancy indicates singleton or twin fetuses *$P < 0.05$; † $P < 0.1$.

plasma E_1S concentration in twin bearing does was about 22.5% greater than those with a single fetus. In the present study, there was no interaction between time (day of blood sampling) and type of kidding on plasma E_1S concentration.

3.4. Relationship of hormones with the reproductive variables

Analyzing the relationship of PAG with other pregnancy hormones such as P4 and E_1S indicated there was a significant positive relationship of PAG and E_1S concentrations during mid-pregnancy. Plasma concentration of PAG was not correlated ($P > 0.05$) with P4 concentration (Table 1). Data regarding associations among PAG, P4 and E_1S concentrations with fetal number, gestation length and birth weight of kids during early- and mid-pregnancy are presented in Table 2. Plasma PAG concentration was positively correlated with fetal number and birth weight of kids during the study period. There was no correlation ($P > 0.05$) among plasma PAG concentrations and gestation length. Inconsistent with this finding, there were inverse relationships of plasma P4 and E_1S concentration with gestation length. Plasma concentration of P4 and E_1S was not associated with the birth weight of kids during early pregnancy (Table 2).

There was a positive correlation of sex of the fetus with plasma concentration of PAG ($r = 0.306$; $P < 0.001$) and E_1S ($r = 0.231$; $P = 0.009$). Sex of the fetus, however, was not correlated with the plasma P4 concentration. Inconsistent with this finding, mean body weight of does was correlated with plasma P4 concentration ($r = 0.244$; $P < 0.001$) but not with the plasma PAG or E_1S

Table 1
Association (correlation coefficients) of plasma pregnancy-associated glycoprotein (PAG) with progesterone (P4) and estrone sulfate (E_1S) during pregnancy in goats.

Variable	Pregnancy period	P4	ES
PAG	Early pregnancy*; n = 75	0.137 (0.199)	0.203 (0.197)
	Mid pregnancy**; n =149	0.009 (0.923)	0.312 (0.009)
	Whole period; n = 224	0.016 (0.818)	0.268 (0.004)

*Early pregnancy = d 26 to d 51 and **mid pregnancy = d 58 to d 114 of gestation.
Values in parenthesis represent corresponding *P*-value.

Table 2
Association (correlation coefficients) of plasma pregnancy-associated glycoprotein (PAG), progesterone (P4) and estrone sulfate (E_1S) with fetal number, gestation length and birth weight of kids during pregnancy in goats.

	Pregnancy period								
	Early pregnancy*			Mid pregnancy**			Whole period		
	Number of fetus	Gestation length	Kid Birth wt.	Number of fetus	Gestation length	Kid Birth wt.	Number of fetus	Gestation length	Kid Birth wt.
PAG (d 28 – d 114)	0.383 (< 0.001)	−0.141 (0.186)	0.537 (< 0.001)	0.471 (< 0.001)	−0.189 (0.079)	0.698 (< 0.001)	0.424 (< 0.001)	−0.153 (0.082)	0.597 (< 0.001)
P4 (d 7 – d 114)	0.307 (< 0.001)	−0.350 (< 0.001)	0.188 (0.071)	0.429 (< 0.001)	−0.403 (< 0.001)	0.339 (< 0.001)	0.310 (< 0.001)	−0.346 (< 0.001)	0.237 (< 0.001)
E_1S (d 7 – d 114)	0.081 (0.396)	−0.324 (< 0.001)	−0.122 (0.220)	0.468 (< 0.001)	−0.532 (< 0.001)	0.321 (0.013)	0.238 (< 0.001)	−0.360 (< 0.001)	0.120 (0.130)

*Early pregnancy = d 7 or d 26 to d 51 of gestation and **mid pregnancy = d 58 to d 114 of gestation.
Values in parenthesis represent corresponding *P*-value.

concentration.

3.5. Multivariate regression analysis

Results of stepwise multivariate linear regression analyzes indicated there was the greatest unstandardized and standardized β coefficient for plasma PAG concentration compared with P4 and E_1S concentration for fetal number (Table 3) and birth weight of kids (Table 4) during pregnancy in goats. Thus, the results indicate that the plasma concentration of PAG is a more precise predictor for both the reproductive variable (i.e., fetal number and birth weight) of kids as compared with plasma P4 and E_1S concentration during early- and mid-pregnancy in goats (Tables 3 and 4). Furthermore, the results of discriminant function analysis indicated d 58 of gestation as the most desirable day for blood sampling for an accurate prediction in this regard. These results provide for an explanation of 100% of the variance and the greatest eigenvalue among all time points when assessments occurred, for prediction of kidding size by evaluating plasma PAG concentrations (S-N value = 2.25 ± 0.36 compared with 3.34 ± 0.15 for single and twin pregnancies, respectively; Table 5).

A receiver operating characteristic (ROC) analysis was performed to evaluate sensitivity and specificity of the PAG assay at

Table 3
Multiple linear regression analyses results for the relationship of plasma pregnancy-associated glycoprotein (PAG), progesterone (P4) and estrone sulfate (E_1S) with fetal number during pregnancy in goats.

Period of pregnancy	Predictor variable	Unstandardized coefficient	Standardized β coefficient	*P*-value	Adjusted R^2
Early pregnancy*	PAG	0.163	0.394	0.008	0.311
	P4	0.074	0.386	0.006	
	E_1S	0.003	0.235	0.086	
Mid pregnancy**	PAG	0.144	0.412	< 0.001	0.428
	P4	0.042	0.296	< 0.001	
	E_1S	0.003	0.335	0.004	
Whole period	PAG	0.121	0.367	< 0.001	0.337
	P4	0.031	0.310	< 0.001	
	E_1S	0.003	0.328	< 0.001	

*Early pregnancy = d 7 or d 26 to d 51 of gestation and **mid pregnancy = d 58 to d 114 of gestation.

Table 4
Multiple linear regression analyses results for the relationship of plasma pregnancy-associated glycoprotein (PAG), progesterone (P4) and estrone sulfate (E_1S) with birth weight of kids.

Period of pregnancy	Predictor variable	Unstandardized coefficient	Standardized β coefficient	*P*-value	Adjusted R^2
Early pregnancy*	PAG	0.195	0.452	0.003	0.328
	P4	0.093	0.337	0.021	
	E_1S	0.005	0.226	0.117	
Mid pregnancy**	PAG	0.848	0.535	< 0.001	0.563
	P4	0.134	0.405	< 0.001	
	E_1S	0.006	0.194	0.048	
Whole period	PAG	0.617	0.427	< 0.001	0.405
	P4	0.089	0.267	0.001	
	E_1S	0.009	0.261	0.002	

*Early pregnancy = d 7 or d 26 to d 51 of gestation and **mid pregnancy = d 58 to d 114 of gestation.

Table 5
Discriminant function analyses results for discrimination of day of blood sampling during pregnancy.

Cononial discriminant function coefficients	Function
at d 58	
Wilks' Lamda value	0.646*
Unstandardized coefficients	−3.489
Function at group centroids	
Kidding size	
Single	−0.644
Twins	0.736
Eigenvalue	0.547
% of variance	100
Canonical correlation	0.60

* $P < 0.05$.

different threshold values (Table 6). Furthermore, the 2.60 value for concentration was identified as the optimum threshold value to differentiate goats with twin fetuses and single fetus with the overall sensitivity and specificity being 85.4% and 70.8%, respectively (Table 6).

4. Discussion

An early identification of pregnancy and number of fetuses after breeding in goat does can result in improvements in reproductive efficiency and pregnancy rate by decreasing inter-kidding interval and providing information for developing an appropriate management system for pregnant does. Thus, an effective method to identify pregnancy status and fetal number may have an important role in further optimization of management practices at goat farms.

In the present study, results indicate that plasma PAG concentration was not associated with P4 concentration during pregnancy

Table 6
Result of ROC analysis [sensitivity (Se), specificity (Sp) and area under curve (AUC)] of pregnancy-associated glycoprotein (PAG) ELISA assessment for determination of either pregnancy or twin fetuses based on different threshold values of circulating PAG concentration (S-N) in goats.

Days of pregnancy	PAG-threshold values	Se (%)	Sp (%)	AUC and *P*-value	95 % CI	
					Lower bound	Upper bound
Pregnancy detection from d 26 until d 114 of gestation	0.20	96.9	75.6	0.945	0.908	0.982
	0.30	94.2	80.0	($P < 0.001$)		
	0.35	92.9	80.0			
Pregnancy detection at d 51 of gestation	0.27	100.0	100.0	1.000	1.000	1.000
	1.15	93.3	100.0	($P < 0.001$)		
	1.90	86.7	100.0			
Determination of twin fetuses from ≥ d 37 until d 86 of gestation	2.45	87.5	66.7	0.831	0.759	0.903
	2.60	85.4	70.8	($P < 0.001$)		
	2.75	75.0	75.0			

CI = confidence interval.

in goats, thus, results of the present study are inconsistent with those reported previously (Lobago et al., 2009). Likewise, there is a lack of association between circulating PAG and P4 concentrations in sheep (Ranilla et al., 1997) and cattle (Karen et al., 2014). The absence of relationship among plasma PAG and P4 concentrations indicate that the production of PAG (mainly from binucleate cells of placenta) and P4 [from corpus luteum (CL)] is, for the most part, independent of each other in goats. Inconsistent with these findings, results of few studies indicate there is a positive relationship between serum PAG and P4 concentrations either during normal (Salve et al., 2016) or abnormal, resulting in failed (Charallah et al., 2010) pregnancies, in goats.

There was a positive correlation ($P < 0.01$) between PAG and E_1S concentrations in the present study. This finding is consistent with those from previous studies where there was a positive association between serum PAG and E_1S concentrations in cattle (Dobson et al., 1993; Lobago et al., 2009). The positive relationship between the circulating concentrations of PAG and E_1S may be due to the common source of production (placental cells).

By using an ELISA system in the present study, there was a gradual increase of PAG concentration in circulation during early pregnancy (from d 26 to d 51). The mean plasma PAG concentration on and after d 28 post-conception (S-N = 0.72–3.39) was substantially greater than the recommended cut-off value for a positive pregnancy diagnosis (S-N = 0.30). This indicates that the ELISA used in the present study could adequately be utilized for pregnancy diagnosis in tropical goat breeds as early as d 28 post-breeding.

The PAG concentration in maternal circulation is maximal on d 51 of pregnancy, irrespective of the fetal number in goats (S-N = 2.34 ± 0.34 and 3.39 ± 0.22, in singleton and twin bearing does, respectively). Thereafter, the concentration remains relatively unchanged throughout the period of mid-pregnancy. The results of the present study are consistent in some ways with those previously reported where the maximal concentration of PAG was at d 48.6 ± 5.0 post-breeding and remained at similar concentrations until 12 wk of gestation in goats (Gonzalez et al., 2000). The continual increase in mean PAG concentration from d 28 to d 51 of gestation observed in the present study is similar to what has been previously reported to occur in goat does (Rovani et al., 2016) where there was a gradual increase in serum PAG concentrations during early gestation that was maximal at about d 62. There was a greater mean concentration of PAG in the present study (S-N = 2.83 ± 0.24 on d 51) than the peak values previously reported (S-N ≈ 1.5; d 62). Furthermore, there was a relatively lesser value for PAG concentration reported in another study with goats (Al-Samawi et al., 2015). The observed difference in PAG values during early gestation in different studies may be partly due to the variation in breed, parity and production level of animals (Lopez-Gatius et al., 2007).

Binucleate cells comprise about 20% of the trophoblast cells and are responsible for production and release of hormones and bioactive products into maternal circulation during gestation (Schlafer et al., 2000). These placental binucleate cells, the major source of maternal PAG, are first observed at d 18 of pregnancy and the relative cell number increases rapidly from d 19 (less than 1–16 per cent) to d 23 (about 22 per cent) of pregnancy (Wango et al., 1990). Based on this observation, it can be expected that a detectable amount of PAG in peripheral blood should be initially detectable around d 24 to d 26 of pregnancy. Thus, the plasma profile of PAG in pregnant goats in the present study (on and after d 26 post-breeding) is consistent with the timing of physiological events related to the placental binucleate cell population during early pregnancy in goats.

There are several factors that may affect the concentration of PAG in circulation during pregnancy in goats and other farm animals. In the present study, there was a greater concentration of plasma PAG in does bearing twin fetuses than does with a singleton fetus. The difference in PAG concentrations was first detectable at d 28 of pregnancy and the pattern was similar until d 93 of gestation. In the present study, the results of greater plasma PAG concentrations in goats bearing twin fetuses compared with a singleton fetus during early- and mid-pregnancy is consistent with results from earlier reports on goats (Batalha et al., 2001), cattle (Szelényi et al., 2015) and sheep (Ledezma-Torres et al., 2006). In some studies, however, there was an effect of litter size on circulating PAG concentration only during mid- and late-pregnancy in farm animals (de Sousa et al., 1999; Szelényi et al., 2015). According to Hayden et al. (1979), total weight of placentomes increases with total fetal weight and hence with the fetal number. Thus, an increase in uteroplacental mass and total binucleate cell population with litter size likely explains the positive relationship of PAG with fetal number and the greater concentrations of plasma PAG in goats bearing multiple fetuses compared to those with a singleton fetus (Echternkamp et al., 2006).

The positive correlation of PAG concentration with number of fetuses and birth weight of kids may partially be due to the consistently greater concentration of plasma PAG in does bearing twin fetuses throughout early- and mid-pregnancy than to the does with a single fetus. Results from the multiple linear regression analyses indicate plasma PAG concentration is the most important predictor for number of fetues and birth weight of kids compared with plasma P4 and E_1S concentrations. On the basis of this information and the result of correlation analyzes, it appears as though it is possible to predict values for two important reproductive variables (i.e., fetal number and birth weight of kids) by assessing the plasma PAG profile in goats. The results of discriminant function analysis indicated that d 58 of pregnancy is the day blood sampling should occur for the most accurate predictions of kidding size by plasma PAG estimation. This may partially be due to there being the greatest difference in mean PAG concentration at d 58 of gestation (S-N difference = 1.09) among does bearing either twin fetuses or a singleton fetus (S- N = 3.34 ± 0.15 vs. 2.25 ± 0.36), compared with other time points during the study.

Plasma P4 concentrations recorded in present study were similar with the data that have been previously reported in goats (Gaafar et al., 2005; Salve et al., 2016 and Yazici et al., 2018) and sheep (Ranilla et al., 1997; Yotov, 2007). Relatively lesser concentrations of blood P4 in pregnant goats, however, have been reported in other studies (Boscos et al., 2003; Haldar et al., 2013). These differences in findings among the studies may be due to difference in the breed, age, management system and the P4 quantitation methods (RIA / ELISA). The results in the present study indicate plasma P4 concentrations remain greater throughout early- and mid-gestation in pregnant does, and these findings are consistent with retrospective findings in goats (Haldar et al., 2013), sheep (Boscos et al., 2003) and cattle (Lobago et al., 2009).

In the present study, there was an effect of fetal number on plasma P4 concentration on and after d 58 of pregnancy. Similarly, Yazici et al. (2018) reported d 51 of gestation as the first time point where there was detection of a significant difference in serum P4 concentration between goat does bearing a single fetus and those bearing twin fetuses. The finding in the present study of a greater P4 concentration in twin bearing does compared with those bearing a single fetus during mid-gestation confirm the results of earlier studies with pregnant does and ewes (Manalu and Sumaryadi, 1998; Yotov, 2007; Haldar et al., 2013). A significant positive correlation between fetal number and diameter of CL was described by Gür et al. (2011). Apart from more CL, a larger size of the luteal tissues in does with multiple fetuses may be partly attributed to the greater circulating concentration of P4 in twin bearing compared with does with a singleton fetus. In the present study, there was a positive correlation between the maternal plasma P4 concentrations and birth weight of kids. This finding is consistent with those from earlier studies with sheep (Manalu and Sumaryadi, 1998) and cattle (Mukasa-Mugerwa and Viviani, 1992).

In the present study, E_1S concentration increases during the period of early gestation (d 22–58 of gestation). The pattern of increase in plasma E_1S concentration is similar to the results from an earlier study Haldar et al. (2013). An increase in blood concentration of E_1S, however, has been reported to occur at d 40–50 and around d 50 of pregnancy in goats (Refsal et al., 1991) and cattle (Hirako et al., 2002), respectively. In the present study, there was an effect of type of kidding (single or twin) on plasma E_1S concentration on and after d 30 of gestation. Similarly, Gür et al. (2011) reported that the concentration of E_1S was affected by number of fetuses and day of gestation after d 50 of pregnancy in ewes.

Though, there was a greater concentration of E_1S in plasma of twin compared to singleton bearing does (on and after d 30 of pregnancy), the inter-individual variation, especially during early pregnancy, is much greater in plasma concentration of E_1S compared with PAG and P4. Likewise, Lobago et al. (2009) reported that the singleton bearing cows had an undulation in E_1S profile as compared with a markedly elevated consistent profile in twin-bearing cows during the pregnancy. These results are suggestive that at an early stage of gestation, measurements of circulating E_1S concentration for pregnancy diagnosis alone may not be a variable that can be reliably used to discriminate singleton and twin fetuses as reported earlier for cattle (Lobago et al., 2009).

Results of the present study on the association of plasma E_1S concentration with values for reproductive variables in pregnant does are in some respects similar to those from previous studies (Refsal et al., 1991; Haldar et al., 2013) where it was reported that there was a positive correlation between plasma E_1S concentrations and number of fetuses does were bearing. The fetal or cotyledonary portion of placentome are the main sources of E_1S that is present in the circulation. Furthermore, the number of cotyledons, total weight of cotyledons and weight of the placenta is greater in does bearing twin fetuses as compared with those bearing a singleton fetus (Ocak et al., 2014). Thus, the difference in these placental characteristics may be the determining factor for the greater ($P < 0.001$) E_1S concentrations in does bearing twin fetuses compared with those bearing a single fetus between d 30 and d 114 of gestation.

In present study, plasma concentration of P4 and E_1S in does was correlated positively with fetal number and inversely with gestation length. A positive correlation between P4 concentration and the number of lambs or kids born has been previously reported (Boscos et al., 2003). In present study, there was a significant positive relationship of plasma E_1S concentration with birth weight of kids only during mid-pregnancy. This finding is similar to that in a study with cattle (Zhang et al., 1999). An absence of significant association of E_1S concentration with duration of gestation and calf birth weight, however, has been reported in Japanees beef cattle (Isobe et al., 2003).

In the present study, the mean birth weight of the singleton fetus was greater than that for twin fetuses. This finding is consistent with earlier findings that the litter size affects birth weight of kids and lambs (Khanum et al., 2001; Juengel et al., 2018; Yazici et al., 2018). Similarly, Savaş (2009) observed that there was a greater birth weight of singleton than twin kids in Turkish Saanen goats. This finding may be explained by the fact that intra-uterine space is of a limited capacity to accommodate offspring during gestation and the dam may not have the physiological capacity to adequately supply multiple fetuses with the increased nutrient demand required for their growth compared with the single fetus. This consequently led to decrease in individual birth weight as the litter size increases (Mellado et al., 2011).

5. Conclusion

The plasma PAG profile was characterized by the gradual increase in concentration during early pregnancy followed by relatively unchanged concentration until d 114 of gestation in does. The effect of fetal number on plasma concentrations of PAG, P4 and E_1S was first observed during early pregnancy that continued throughout mid-gestation. During mid-pregnancy, does bearing twin fetuses have a greater mean plasma concentrations of all three pregnancy hormones (i.e., PAG, P4 and E_1S). The relationship of fetal number and birth weight of kids during early pregnancy was limited to the plasma PAG concentration. Overall, the plasma profile of PAG, P4 and E_1S were affected by the fetal number and birth weight of kids (only PAG). Through statistical analyzes, plasma PAG was identified as the best predictor variable for fetal number and birth weight of kids compared with the plasma concentrations of P4 and E_1S. Furthermore, d 58 of gestation was found to be the most appropriate single time point of blood sampling for prediction of kidding size using plasma PAG as a biomarker in goats.

Conflicts of interest statement

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Disclosure statement

Authors have nothing to disclose.

Acknowledgements

The study was supported by a grant from SERB-DST, Govt. of India, New Delhi (**Grant No**.: YSS/2015/001518). The authors are thankful to the support extended by the Director of the Institute for providing necessary facilities to carry out this study. The skilled technical assistance by Mr. H. A. Himkar in USG and help from Mr. Ranjeet Singh for collection of blood samples are duly acknowledged.

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