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Changes in milk and plasma progesterone and pregnancy-associated glycoprotein and their relationships with the foetal number during early pregnancy in Jakhrana goats

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ABSTRACT

This study was designed to investigate milk and circulating profiles of progesterone (P4) and pregnancy-associated glycoprotein (PAG) during early pregnancy and to determine their associations with the foetal numbers in Jakhrana goats. For this, milk (whole and defatted milk) and blood samplings from10 pregnant and 3 non-pregnant Jakhrana goats were continued from the day 7 until day 51 post-mating. The PAG profile in both milk and plasma increased gradually from day 26 to 51 of gestation. Whereas, circulating P4 remains unchanged during early pregnancy in pregnant goats. The P4 and PAG concentrations in blood plasma, whole milk and defatted milk were 1.30, 1.24 and 2.04, 1.98, 1.99 and 1.88 folds higher in twin foetus than the single foetus bearing does, respectively. The P4 and PAG in plasma and milk samples were positively correlated with the foetal number. However, the results of stepwise-multivariate linear regression analyses indicated milk and circulating P4 as better predictors of foetal numbers than plasma PAG. The defatting of milk samples resulted in about 2-folds decline in the P4 concentration, however, the PAG level remains unaffected. In conclusion, milk and plasma profiles of P4 and PAG were affected by the foetal number and P4 is a reliable predictor of foetal number during early pregnancy in Jakhrana goats.

Keywords: Early pregnancy, Progesterone, Pregnancy-associated glycoprotein, Jakhrana goat

Early diagnosis of pregnancy is a key factor for the reproductive management and profitability of goat farming. An accurate and suitable method for pregnancy diagnosis provides option for timely re-breeding or culling of non-pregnant animals (Karadaev 2015) and proper nutritional management of animals during gestation (El Amiri *et al.* 2015). In farm animals, the ovaries and foeto-placental unit secrete several biochemical and endocrine biomolecules into the blood circulation of dam (Hoffmann and Schuler 2002). Among these, progesterone (P4) and pregnancy-associated glycoprotein (PAG) are two important molecules of gestation, that can be used for the determination of pregnancy and the number of foetus in goats.

Detection of PAGs in blood circulation in ruminant is used pregnancy biomarker and to identify the status of foeto-placental unit in farm animals (Wooding *et al.* 2005). The association of foetal number with milk and plasma P4 (Sausa *et al.* 1999) and PAG (Haldar *et al.* 2013) is demonstrated earlier in goats. The Jakhrana is a large size important dairy goat breed of India with a compact body, predominantly black coat colour and typical white speckles on the ears (Rai and Singh 2005). However, information about the relative importance of milk and circulating P4 and PAG concentrations for prediction of the number of foetus and gestation length in Jakhrana and other tropical goats is scarce.

Keeping this background in mind this investigation was designed in Jakhrana does to determine the time dependent changes in P4 and PAG concentrations in milk and peripheral blood with single or twin foetuses; and investigate the association and predictive importance of P4 and PAG concentrations with the number of foetuses and gestation length.

MATERIALS AND METHODS

Experimental animals: This study was conducted at ICAR-Central Institute for Research on Goats (ICAR-CIRG), Makhdoom, Mathura, Uttar Pradesh, India in thirteen pluriparous (3–4 parity) healthy and lactating Jakhrana goats. During the breeding season (March to May), the oestrus was recorded twice a day (morning and evening), and does were bred about 10–12 h after the beginning of oestrus. All the experimental procedures carried out were in accordance with good veterinary practices and approved by Animal Ethics Committee of the Institute.

Detection of pregnancy: Ultrasonography (USG) was performed at day 37 after mating with the help of an ultrasound scanner fitted with a real-time convex array

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trans-rectal transducer (PVF-738F) using adjustable frequency between 5–7 MHz. The gestation lengths were recorded at delivery.

Collection and storage of milk and blood samples: The milk (~35 mL) and blood samples (6 mL) were collected from day 7 until day 30 post-mating at every 4th days followed by at a weekly interval until day 51. The sampling schedule for non-pregnant goats was similar to the pregnant goats. The blood samples from the jugular vein were collected into K2-EDTA Vacutainer tubes (Becton Dickinson, BD, Franklin Lake, NJ, USA). The blood cells were precipitated by centrifugation at 2,500×*g* for 10 min, (at 4°C) and the supernatant plasma samples were collected and stored at –30°C until further analysis.

The whole milk samples were collected into the vials with bronopol (50 µL of 18% solution) as a preservative. Approximately half volume of milk samples was centrifuged at 5000×g for 20 min at 4°C and the defatted milk was harvested. The defatted and whole milk samples were kept at –30°C until analysed.

P4 assay: The P4 concentration in plasma and milk (whole and defatted) samples was quantified using commercial ELISA kits (DRG Diagnostic, Germany) following manufacturer's instructions and the protocol described previously by Singh *et al.* (2019a) with minor modifications. Briefly, 25 µL of control, standard, and test samples were added into the antibody coated microtiter plate and incubated at room temperature (RT) for 5 min. To it, 250 µL of enzyme conjugate was added into each well, plates were sealed and incubated for 1 h at 37°C. After washing four times (wash buffer provided with the kit) using microtiter plate washer (Hydroflex, Tecan, Austria), 150 µL substrate solution was dispensed to each well and incubated for 20 min in dark at RT. The reaction was stopped by addition of 50 µL stop solution (0.5 M H_2SO_4) and the absorbance was determined immediately at 450 nm (Sun Rise, Tecan, Austria).

The P4 concentration in milk samples was determined as plasma samples with minor modifications, considering the difference in the matrix of plasma and milk samples. Briefly, 20 µL of standard, control and test samples were pipetted into the 96 well microtiter plates and incubated for 5 min at RT. Thereafter, enzyme conjugate (200 µL) was added plate was mixed for 1 min. Following 60 min incubation at RT, plates were washed five times and 200 µL of substrate solution was added before 15 min incubation at RT in dark. Finally, stop solution (50 µL) was added and absorbance was determined at 450 nm. Prior to the assessment of the P4 level in goat milk and plasma samples with the human P4 kit, dilutional linearity was determined compatibility of ELISA systems with the sample matrix.

PAG assay: The milk (whole and defatted) and plasma PAG levels were determined by sandwich ELISA as recommended by the manufacturer (IDEXX Laboratories, Westbrook, ME, USA) as described earlier (Singh *et al.* 2019b). Briefly, the control and test plasma samples (100 µL) along with sample diluents (25 µL) were added into the 96-well antibody coated plate and then incubated for 60 min at 37°C without shaking. The non-reactants from the wells were removed by 4 times washings (wash buffer provided with the kit) and then 100 µL detector solution was added and plates were incubated at RT for 30 min. Subsequently, wells were washed four times, and then 100 µL of the conjugate solution was added and incubated for 30 min at RT. After the end of incubation, 100 µL substrate solution was added and incubated for 15 min at RT, the reaction was stopped (provided by manufacturer) and absorbance was recorded.

The PAG in milk samples were determined with certain modifications of the above protocol. Briefly, 150 µL each of control (positive and negative) and milk samples (whole or defatted milk) were added into 96 well plates and incubated for 1.5 h at 37°C with shaking at 250 rpm (Digital Multi-MicroPlate Genie Pulse, SI-4000A, USA). After washing and incubation with detector solution, conjugate solution (100 µL) was added. After 30 min incubation at RT and four washings, substrate solution (100 µL) was added and then incubated in dark for 20 min. After adding stop solution (100 µL), absorbance was measured at 450

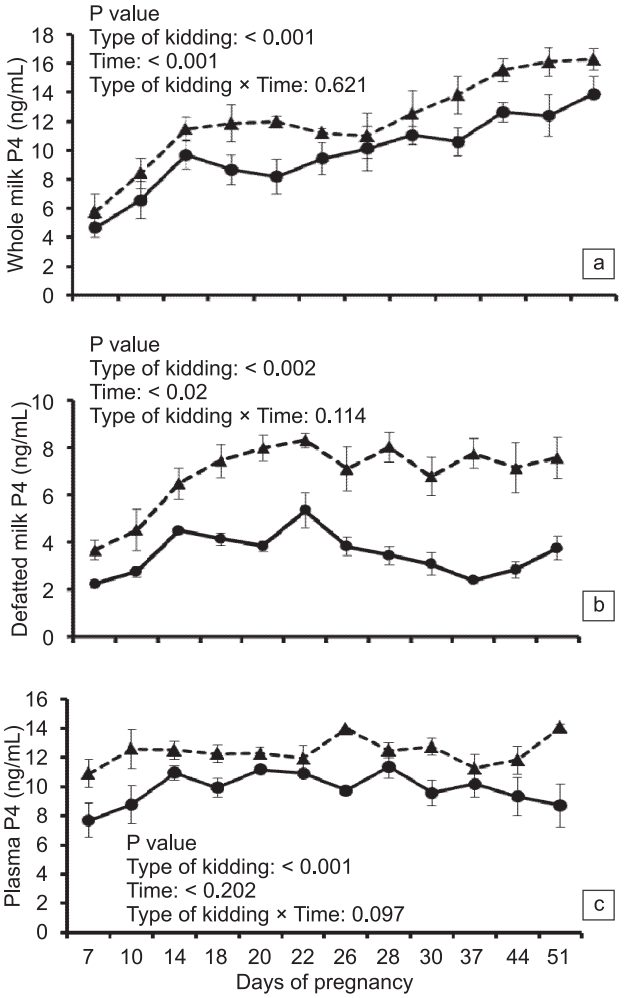

Fig. 1. Concentration of progesterone (P4) in (a) whole (b) defatted milk and (c) blood plasma of singleton (●) or twin (▲) foetuses bearing Jakhrana does.

nm. The outcome was determined for each sample following the process described earlier (Singh *et al.* 2019b).

Statistical analyses: The relationship of milk and plasma P4 and PAG concentrations, with foetal number and gestation length were determined by Pearson's correlation coefficient. The method of 'Linear Mixed-Model' was used to estimate the effect of 'Treatment' (number of foetus) and 'Time' (days post-breeding). A stepwise multiple-linear regression method was used to detect independent predictor of foetal numbers, as described earlier (Singh *et al.* 2019b).

RESULTS AND DISCUSSION

On the basis of USG results, 10 goats were pregnant [(equal number of animals (n = 5) had either single or twin foetuses)] and 3 does were non-pregnant. The average gestation period of the does was 149.4±0.93 days for single foetus and 145.0±0.32 days for twin foetus.

Milk and plasma P4 profile: The P4 level during early pregnancy was low (p<0.01) in single vs twin foetus in whole milk (8.85±0.43 vs 12.02±0.48 ng/mL), defatted milk (3.45±0.17 vs 7.31±0.22 ng/mL) and in plasma (9.92±0.24 vs 12.45±0.21 ng/mL) samples (Fig. 1). Overall, 1.36-folds higher P4 was observed in milk and plasma sample of does bearing twins than does with single foetus. The P4 concentration in plasma, defatted milk and milk fat was positively correlated (p<0.01) with the foetal number (Table 1). The P4 concentration in plasma was about 2.1-folds higher than in the defatted milk samples (11.16±0.20 vs 5.32±0.23 ng/mL; p<0.001). The P4 concentration in plasma and whole milk samples were similar (p>0.05). About 1.97-folds reduction in P4 concentration was observed after removal of the fat (defatted milk) from the milk samples [10.49±0.35 (whole milk) vs 5.32±0.23 ng/mL (defatted milk)]. The results demonstrate that the plasma P4 was not associated with plasma PAG in goats. Similarly, an absence of the relationship among circulating P4 and PAG concentration is reported earlier in cattle (Karen *et al.* 2014).

Table 1. Pearson correlation coefficients (r) of milk and plasma progesterone (P4) and pregnancy-associated glycoprotein (PAG) with foetal number and gestation length (days) during early pregnancy in Jakhrana goats

Variable	Number of foetus	Gestation length
P4		
Whole milk	0.425	–0.367
	p<0.01	p<0.01
Defatted milk	0.536	–0.522
	p<0.01	p<0.01
Plasma	0.601	–0.542
	p<0.01	p<0.01
PAG		
Whole milk	0.337	–0.357
	p<0.01	p<0.01
Defatted milk	0.290	–0.320
	p<0.05	p<0.05
Plasma	0.531	–0.425
	p<0.01	p<0.01

We observed that the plasma and milk P4 concentration remain significantly higher in the pregnant goats than non-pregnant goats during the early gestation. These findings are similar to the earlier reports in sheep (Roberts *et al.* 2017) and cattle (Lobago *et al.* 2009). The observed a significant effect of foetal number on plasma and milk (whole and defatted) P4 concentration during the early gestation period is also reported in ewes (Gür *et al.* 2011) due to significant positive correlations between foetal number and size of corpus luteum. Therefore, a greater number and larger size of CL might result into a higher concentration of circulating and milk P4 in does with twin foetuses compared to the single foetus bearing Jakhrana goats.

Milk and plasma PAG profile: In pregnant goats, the mean PAG concentrations in whole milk (4.5 folds; 0.94±0.25 vs 0.21±0.09 folds), defatted milk (4.8 folds; 0.96±0.02 vs 0.20±0.05 folds) and blood plasma (10.1folds; 1.92±0.30 vs 0.19±0.08) were significantly (p<0.01) higher

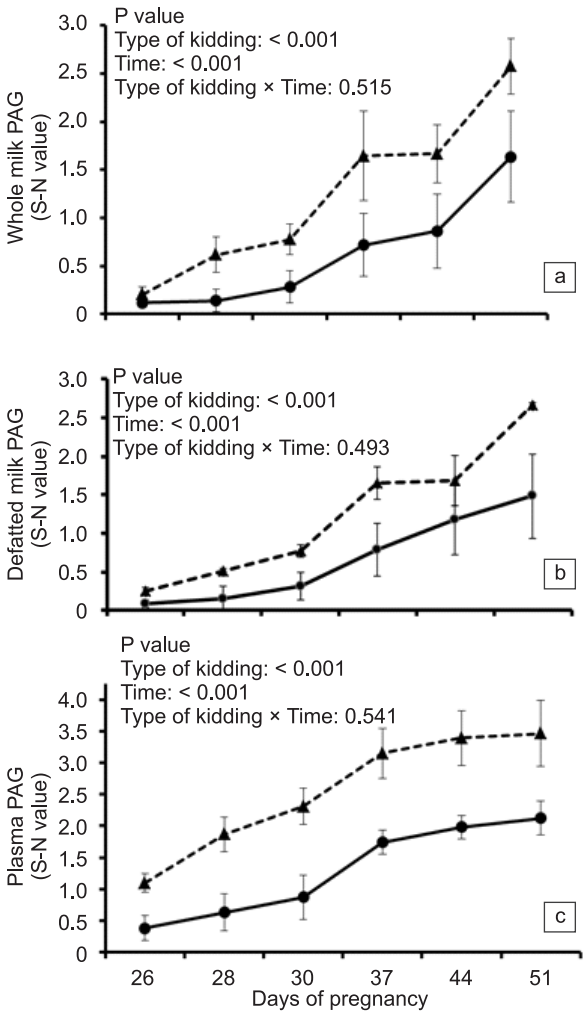

Fig. 2. Pregnancy-associated glycoprotein (PAG) in (a) whole (b) defatted milk and (c) plasma of singleton (●) or twin (▲) foetuses bearing Jakhrana does.

Table 2. Multiple linear regression analyses for demonstration of relationship of progesterone (P4) and pregnancy-associated glycoprotein (PAG) in milk and plasma with the number of foetuses during early pregnancy in Jakhrana goats

	Predictors	Unstandardized coefficient	Standardised β coefficient	P-value	Adjusted R^2
Whole milk	P4	0.071	0.501	<0.001	0.278
	PAG	0.048	0.090	0.491	
Defatted milk	P4	0.120	0.483	<0.001	0.286
	PAG	0.107	0.210	0.070	
Plasma	P4	0.177	0.560	<0.001	0.569
	PAG	0.124	0.420	<0.001	

than the non-pregnant goats. The concentration of PAG in milk (whole and defatted) and plasma gradually increased and reached to the highest concentration at day 37 of gestation in goats with singleton and twin pregnancies (Fig. 2a,b,c). In the twin bearing goats, overall PAG concentration was 1.77-folds (defatted milk: 0.67±0.16 vs 1.19±0.20), 1.89 folds (whole milk: 0.63±0.15 vs 1.19±0.18) and 1.98-folds (plasma samples: 1.29±0.19 vs 2.55±0.19) higher than singleton bearing goats. About 2.1-folds higher PAG concentration was observed in plasma compared with the whole and defatted milk samples. Removal of fat from the whole milk did not affect the PAG concentration. This may be because the PAG present in milk samples is not soluble in milk fat.

Relationship of P4 and PAG with reproductive variables: The correlation analyses indicated positive associations among the P4 and PAG concentrations of plasma ($r=0.199$, $p>0.05$), defatted milk ($r=0.215$, $p>0.05$) and whole milk ($r=0.494$, $p<0.01$) samples. The PAG concentration of plasma and milk (defatted and whole milk) was positively correlated with gestation length (Table 1). The whole milk P4 was positively correlated to the gestation length ($r=0.565$; $p<0.01$). In contrast to this, no association between plasma P4 and gestation length was observed.

According to the outcomes of the present study, does with twin foetuses have greater milk and plasma PAG concentration compared to the does with singleton foetus. The higher PAG in milk and circulation might be due to the more number of placental mono- and bi-nucleate cells in twin bearing goats compared to the does with a singleton. The results of linear regression analysis demonstrate higher unstandardized and standardised β coefficient for milk and plasma P4 as compared to PAG for the foetal number in goats (Table 2). Thus, P4 could be considered as the reliable predictor variable for the foetal number in Jakhrana goats.

The plasma and milk P4 concentration remain significantly higher in the pregnant goats than non-pregnant goats, whereas, the PAG concentration gradually increased and reached to the highest concentration at d 37 of gestation. The defatting of milk samples resulted in about 2-folds decline in the P4 concentration but not in the PAG concentration. The P4 and PAG concentrations in plasma and milk samples of the twin foetuses bearing does were significantly higher compared to the single foetus bearing goats. The milk and plasma concentrations of P4 were the reliable predictor variable for foetal number and gestation length in Jakhrana goats.

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