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Pregnancy-associated glycoproteins concentrations during early gestation in pregnant Awassi sheep

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Summary

This study was aimed at determining the serum pregnancy-associated glycoproteins (PAG) profile of pregnant Awassi sheep during early gestation. A total of 122 Awassi ewes were used, which were in days 28-56 (28, 30, 32, 36, 42, 49, and 56) of gestation. Blood samples were taken via jugular venepuncture. Out of these animals, 104 ewes that had delivered (singleton lambs, n = 60; twin lambs, n = 44), according to lambing records, were selected for the assessment of serum PAG levels. The serum PAG levels was measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit (IDEXX Bovine Pregnancy Test). Whereas serum PAG levels (mean ± standard error) on days 28, 30, 32, 36 and 42 of gestation were found to be similar (0.237 ± 0.031, 0.402 ± 0.067, 0.329 ± 0.036, 0.397 ± 0.075, and 0.409 ± 0.060, respectively), the levels measured on days 49 and 56 of gestation were significantly higher (0.712 ± 0.088 and 0.798 ± 0.115, respectively) ($p < 0.05$). The serum PAG levels of the twin-pregnant ewes on days 28, 30 and 42 of gestation were higher than those of the single-pregnant ewes (twin-pregnant ewes: 0.314, 0.648 and 0.594, respectively; single-pregnant ewes: 0.186, 0.258 and 0.308, respectively; $p < 0.05$). With a cut-off sample minus negative (S–N) value set at > 0.300, the sensitivity of serum PAG level-based pregnancy diagnosis was 51.95% between days 28 and 42 of gestation and 92.69% between days 49 and 56 of gestation. On the other hand, with a cut-off S–N value set at > 0.100, the sensitivity of the PAG ELISA test was 94.81% between days 28 and 42 of gestation and 100% between days 49 and 56 of gestation. In conclusion, the serum PAG levels of the pregnant Awassi sheep remained similar between days 28 and 42 of gestation, but progressively increased thereafter. The serum PAG levels of the twin-pregnant Awassi ewes were significantly higher than those of the single-pregnant sheep on days 28, 30 and 42 of gestation, but they were not different on days 32, 36, 49 and 56 of gestation.

Keywords: Awassi, pregnancy, pregnancy-associated glycoproteins, sheep

While ewes have an average of 6-9 oestrus cycles during the breeding season, the length of the mating period is limited to 2 or 3 cycles in order to facilitate flock management during the lambing season and to raise uniform lambs for butchery. The prolongation of the mating period prolongs the lambing period, which increases the occurrence of problems (21). In order to increase the pregnancy rate and lamb yield of flocks, open ewes need to be identified as quickly as possible and re-mated in the intended mating period. Thus, early pregnancy diagnosis has become a highly important tool for both reproductive management and profitability in sheep production (10).

The most commonly used method in the diagnosis of early pregnancy in sheep is transrectal or transabdominal ultrasonography (5, 8). While offering a high level of accuracy in the diagnosis of pregnancy in sheep, ultrasonography has several practical limitations. Some

of these limitations are related to the transducer type and frequency, the availability, portability and cost of ultrasonography equipment and the construction of handling facilities. Furthermore, animals need to be fasted for at least 8 hours prior to transrectal ultrasonography, their rectum should be emptied before examination, and they should be maintained in dorsal recumbency during examination. On the other hand, transabdominal ultrasonography requires the shaving of the ventral abdomen, particularly in heavily wooled sheep breeds. All these procedures are both time-consuming and laboursome (3, 18, 27).

Pregnancy-associated glycoproteins (PAG) originate from the placenta and are produced by the mononucleate and binucleate cells of the ruminant trophectoderm throughout gestation (25). The detection of PAG in maternal circulation is considered a good indicator of pregnancy in ruminants, including cattle (23), sheep (1, 7), goats (24) and reindeer (22). The close similarity of the molecular structure of bovine and ovine PAG (30) makes it possible to use bovine PAG ELISA tests in sheep (4, 26). The variability of the plasma PAG profile among sheep breeds (13, 14, 16) requires the determination of breed-specific standard PAG profiles for an accurate PAG-based pregnancy diagnosis. This study was aimed at determining serum PAG profiles of pregnant Awassi sheep during gestation (between days 28 and 56).

Material and methods

Animal management. This study was approved by the Local Ethics Committee for Animal Experiments of Harran University. The study was carried out on 122 Awassi sheep aged 2-5 years and weighing 40-60 kg from June to December 2019 at a rural farm in the Sanliurfa province, in the South-eastern Anatolia Region of Turkey. All ewes had access to pasture (wheat stubble) during the summer and were fed additional concentrate. Teaser rams were used twice daily (in the morning and evening) for oestrus detection. Ewes showing oestrus were hand-mated with breeding rams. The ewes included in this study were mated for the first time in the breeding season. The mating and lambing records of all ewes were maintained in the herd management software of the enterprise.

Experimental design. Using the breeding records kept at the farm, the animals included in the study were divided into 7 groups according to post-mating (PM) days (PM–28, PM–30, PM–32, PM–36, PM–42, PM–49, PM–56). Groups 1 (n = 15), 2 (n = 19), 3 (n = 12), 4 (n = 14), 5 (n = 17), 6 (n = 13) and 7 (n = 14) were formed from animals on post mating days 28, 30, 32, 36, 42, 49 and 56, respectively. Blood samples were collected via jugular vein puncture into 10 ml plain vacutainer tubes (Hema Tube, Ankara, Turkey). Sera were separated by centrifugation at 3200 g for 10 minutes and stored at –18°C until PAG analysis.

Making use of lambing data, serum samples from 104 ewes that had lambed were analysed for PAG levels. Serum PAG analyses were performed using a commercial ELISA kit (IDEXX Bovine Pregnancy Test Kit, Westbrook, Maine, USA). The ELISA test was administered by experienced technical staff in accordance with the manufacturer's instructions. Using anti-PAG monoclonal antibody-coated plates, 100 µl samples and assay controls (2 positives and 2 negatives) were incubated (37°C, 60 minutes) and washed 4 times with a wash buffer. This was followed by the addition of the detector solution, incubation at room temperature for 30 min and 4 washes. Next, the conjugate solution was added, incubation was performed at room temperature for 30 min, and another 4 washes were performed. Subsequently, the substrate solution was added, incubation was performed at room temperature for 15 min, and at the end of the incubation period the stop solution was added. The results were read on a spectrophotometer at a wavelength of 450 nm. The S–N (sample minus negative) value of each sample was determined by subtracting the mean value of the optic densities (OD) of two negative controls from the OD of the sample. As the PAG levels in the samples were correlated with the colour intensity, the S–N values determined in this study were used as the serum PAG levels of the samples.

Statistical analysis. The normality analysis of the data was performed using skewness and kurtosis. Descriptive statistics were generated for the groups. The results were given as mean ± standard error of mean (SEM). The level of difference between the groups was ascertained by Tukey's test and variance analysis. Sensitivity was defined as the proportion of lambed ewes correctly detected as pregnant by the PAG ELISA using of > 0.100 or > 0.300 PAG S–N cut-off levels. Statistical calculations were performed using the Statistical Package for Social Sciences (SPSS) version 24.0 software. The statistical significance level was set at $p < 0.05$.

Results and discussion

The lambing results (open, singleton, twin) of the 122 ewes included in the study are presented in Table 1. The mean serum PAG S–N levels of the ewes determined on different days of early gestation are presented in Table 2. The serum PAG S–N value of one ewe in PM–36 was not included in statistical calculations, because it was very high (S–N: 1.732). The mean serum PAG levels of the ewes in PM–28 were lower than those of the ewes in PM–49

Tab. 1. Distribution of open, single-pregnant and twin-pregnant ewes in the study groups

	Groups							Total (n)
	PM–28	PM–30	PM–32	PM–36	PM–42	PM–49	PM–56	
Twin-pregnant (n)	6	7	6	8	6	6	5	44
Single-pregnant (n)	9	12	6	6	11	7	9	60
Open (n)	6	2	3	2	3	0	2	18
Total (n)	21	21	15	16	20	13	16	122

Explanation: PM – post-mating

Tab. 2. Mean PAG S–N values of pregnant Awassi sheep on different days of early gestation

Groups	n	Mean ± st. error	95% confidence interval for mean		Minimum	Maximum
			Lower bound	Upper bound		
PM–28	15	0.237 ± 0.031[a]	0.170	0.304	0.049	0.472
PM–30	19	0.402 ± 0.067[a]	0.260	0.543	0.036	1.261
PM–32	12	0.329 ± 0.036[a]	0.251	0.408	0.182	0.531
PM–36	14	0.397 ± 0.075[ac]	0.233	0.561	0.076	1.151
PM–42	17	0.409 ± 0.060[ac]	0.282	0.536	0.107	1.020
PM–49	13	0.712 ± 0.088[bc]	0.520	0.903	0.433	1.563
PM–56	14	0.798 ± 0.115[b]	0.550	1.045	0.143	1.615

Explanations: PAG – pregnancy-associated glycoproteins; S–N – sample minus negative; PM – post-mating; different superscripts letters represent statistically significant difference between the groups; $p < 0.05$

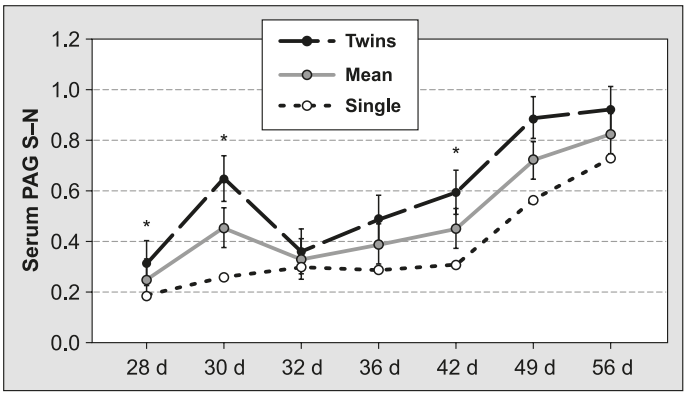

Fig. 1. The serum pregnancy-associated glycoprotein (PAG) profiles of twin-pregnant and single-pregnant Awassi ewes from day 28 to 56 of gestation

Explanation: * indicates statistically significant differences between the single- and twin-pregnant ewes; $p < 0.05$

and PM–56 ($p < 0.001$). Furthermore, the mean serum PAG levels of the ewes in PM–30 were also lower than those of the ewes in PM–49 ($p < 0.05$) and PM–56 ($p = 0.002$). Likewise, the mean serum PAG levels of the ewes in PM-32 were lower than those of the ewes in PM–49 ($p < 0.05$) and PM–56 ($p = 0.001$). The mean serum PAG levels of PM–36 tended to be lower than those of PM–49 ($p = 0.068$) and they were lower than those of PM–56 ($p=0.005$). Moreover, the mean serum PAG levels of PM–42 tended to be lower than those of PM–49 ($p = 0.057$) and were also lower than those of PM–56 ($p = 0.003$).

The mean serum PAG levels of the twin-pregnant ewes were significantly higher than those of the single-pregnant ewes in groups PM–28, PM–30 and PM–42, ($p < 0.05$); these levels tended to be higher in Group PM–49 (Fig. 1, $p = 0.65$).

In the present study, 65 of the ewes analysed for serum PAG levels had serum PAG S–N levels higher than 0.300, 35 ewes had serum PAG S–N levels within the 0.100–0.300 range, and 4 ewes had serum PAG S–N levels lower than 0.100 (Tab. 3). Among the ewes with low serum PAG S–N levels (< 0.300), 94.87% (n = 37) were in days 28-42 of gestation, and 5.13% (n = 2) were in days 49-59 of gestation. Furthermore, 23.1% (n = 9) of the ewes with low serum PAG S–N levels gave birth to twin lambs.

In sheep production, early pregnancy diagnosis is an indispensable management tool for the reproductive planning of open ewes, the prediction of the lambing rate and the development of an appropriate nutritional programme (6, 16). The detection of PAG, which are placental products, can be used as an alternative method for early pregnancy diagnosis. The variability of serum PAG profiles among sheep breeds may affect the results of pregnancy diagnosis. The present study investigated the serum PAG profiles of Awassi sheep in the early phase of pregnancy (days 28-56 of gestation).

In this study, we observed that serum PAG levels remained similar between days 28 and 42 of gestation, and significantly increased as of day 49. Previous studies have pointed out the variability of serum PAG profiles among sheep breeds. Ledezma-Torres et al. (11) determined that, in German black headed mutton sheep (GBM), Rhoen sheep and GBM X Dorper crosses, plasma PAG levels started to increase between weeks 3 and 4 of gestation, increased gradually until week 9, remained the same between weeks 9 and 19, and made a peak shortly before parturition. Furthermore, serum PAG1 levels have been reported to increase continuously throughout gestation in Polypay X Dorset crosses (19), as

Tab. 3. Sensitivity (Se) of pregnancy results based on PAG S–N levels during early gestation

Groups	Day of gestation	S–N < 100 (n)	S–N = 100-300 (n)	S–N ≥ 300 (n)	Se cut-off > 300 (%)		Se cut-off > 100 (%)	
PM–28	28	2	8	5	33.3		86.67	
PM–30	30	1	8	10	52.6		94.70	
PM–32	32	0	6	6	50.0	51.95	100.00	94.81
PM–36	36	1	5	8	57.1		92.86	
PM–42	42	0	6	11	64.7		100.00	
PM–49	49	0	0	13	100.0	92.59	100.00	100.00
PM–56	56	0	2	12	85.7		100.00	
Total		4	35	65	62.5		96.15	

Explanation: PM – post-mating

well as in Konya Merino (29) and Texel (20) breeds. On the other hand, El Amiri et al. (20), ascertained that in Moroccan sheep (Boujaad and Boujaad X D'man) serum PAG levels varied in different weeks of gestation. Moreover, there are reports indicating decreased serum PAG levels on day 42 of gestation in Konya Merino (9) and Lacaune sheep (7). A similar change was also observed in cattle (17).

It is suggested that the foetal number can be determined on the basis of PAG levels in the maternal circulation (3, 15). Previous research demonstrated that circulating PAG levels during early gestation are higher in multiple-pregnant ewes compared to single-pregnant ewes (6, 15, 19). In the present study, statistically significant differences were detected between the twin-pregnant ewes and single-pregnant ewes for serum PAG levels measured on days 28, 30 and 42 of gestation. These differences tended to decline on day 49 and disappeared by day 56 of gestation. In their study on Kangal-Akkaraman sheep, Alkan et al. (7) report that while the serum PAG levels measured on days 40, 45 and 50 of gestation were higher in twin-pregnant ewes compared to single-pregnant ewes, no difference was found between these ewes on days 55 and 60 of gestation. The higher circulating PAG levels of twin-pregnant ewes are attributed to the higher number of placentae, and hence the higher number of binucleate cells in the trophectoderm and the larger area of placental attachment (3, 15, 19).

While the manufacturer of the PAG ELISA test used in the present study recommends the test for sheep after day 35 of gestation, there are reports indicating the possibility of its use in the earlier phase of gestation (4). In the present study, 37.5% (39/104) of the pregnant ewes tested for serum PAG levels showed S–N values below 0.300, which is the cut-off value for pregnancy diagnosis (Tab. 3). Similar results were reported for the Konya Merino in a study in which the sensitivity of the bovine PAG ELISA test was found to be low before day 56 of gestation (9). The sensitivity of the bovine PAG ELISA test used in the present study was found to be lower than previously reported for the same test in early gestation (2, 6) and at any time during gestation as of day 33 post-mating (20). The family of bovine PAG genes is composed of a minimum of 22 transcribed genes and several variants. The PAG family members are classified into "ancient" and "modern" groups based on phylogenetic analyses. The family members show sequence differences. They also exhibit clearly distinctive spatiotemporal distribution and relative levels of expression. The most common boPAG transcript is boPAG-2 (28). The PAG ELISA kit can detect only several members of this large PAG gene family that are most abundant in bovine placenta (PAG-4, PAG-6, PAG-9, PAG-16, PAG-18 and PAG-19) (12). Differences in PAG levels observed between sheep breeds could be related to the fact that the PAG produced by local sheep breeds differ from the types of PAG detected by the bovine PAG ELISA test used in the present study. Furthermore, the secretion pattern of different PAG molecules may vary among sheep breeds.

The variability of serum PAG levels among sheep breeds (16) would decrease the reliability of pregnancy diagnosis based on the cut-off value of PAG S–N 0.300. In the present study, at a PAG S–N cut-off value of > 0.100, the sensitivity of the pregnancy diagnosis test increased significantly (Tab. 3). Therefore, there is a need for further studies to compare the performance of PAG ELISA tests between different ewe breeds and to determine breed-specific cut-off values. Sheep have seasonal breeding patterns, and their mating period is limited (21). Therefore, to increase the pregnancy rate and productivity of sheep, open ewes should be identified using the low PAG S–N cut off value in early pregnancy and they should be re-mated during the limited mating period.

In conclusion, the serum PAG levels of Awassi sheep remained similar between days 28 and 42 of gestation, but showed a continuous increase with the advance of gestation thereafter. Until day 42 of gestation, the serum PAG levels of 37% of the pregnant ewes were below an S–N cut-off value of 0.300. With the S–N cut-off value set at ≥ 0.100 instead of ≥ 0.300, the sensitivity of the PAG ELISA test between days 28 and 42 increased from 51.95% to 94.81%. The fact that the serum PAG levels of the twin-pregnant ewes were significantly higher than those of the single-pregnant ewes on days 28, 30 and 42 of gestation, but showed no difference from those of the single-pregnant ewes on days 32 and 36 of gestation, should be taken into consideration when diagnosing twin pregnancies.

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