Determining the fetal number to avoid pregnant sheep management, feeding, and delivery issues is of vital importance. This study aimed to determine the levels of pregnancy-associated glycoprotein (PAG) and pregnancy-specific protein B (PSBP), which are pregnancy proteins, to accurately predict singleton and twin pregnancies in Awassi sheep. A total of 40 Awassi sheep were used for the study. According to the number of offspring, pregnant ewes were separated into two groups. The study’s first group (Group 1) included singleton pregnant ewe (n = 20), while the second group (Group 2) included twin pregnant ewe (n = 20). Blood samples were collected from the ewes of both study groups at 30th, 45th, 60th, 75th and 90th day of gestation, which were used for PAG and PSBP evaluation along with ultrasonographic examination. Independent samples t-test and repeated measurements ANOVA were used to analyze the data. The correlations between the measures were calculated using Pearson correlation coefficients. Accordingly, a statistically significant difference was observed between single and twin pregnant sheep for all PAG and PSBP measurements at days 30-45-60-75 and 90 (p < 0.05). In singleton and twin pregnant ewes, there was a statistically significant difference in PAG and PSBP measurements (p < 0.05). In singleton pregnant ewes, a significant positive correlation was found between PAG30 and PSBP30 values (p < 0.05), while a significant negative correlation was found between paired measures of PAG45-PSBP90, PAG60-PSBP90, and PAG75-PSBP90 (p < 0.05). In conclusion, significant differences in pregnancy protein levels were found in singleton and twin pregnant ewes. It was deduced that knowledge of this difference might give sheep breeders an idea about management factors.

Keywords: sheep, twinning, pregnancy-associated glycoprotein, pregnancy-specific protein B
plasma progesterone levels in multiple pregnancies are significantly higher than in singleton pregnancies (32). The disadvantages of the method include the high cost of progesterone analysis, the long process, the need for a laboratory, and the limited accuracy in separating singleton from multiple pregnancies. (41).

In sheep, pregnancy proteins (PAG and PSBP) are of placental origin (55) and can be present in the maternal circulation from the 3rd week of gestation until the 2-3rd week postpartum (31, 54). PAG, widely used in early pregnancy diagnostics, is used to detect embryonic/fetal death, identify twin pregnancies, and monitor placental functions (10, 25, 30). PAG is identified in many sheep breeds after the third or fourth week of pregnancy. Its level rises gradually from the 3rd to the 9th week of pregnancy, then rises again from the 17th to the lambing week, with variability between the 9th and 17th weeks. It decreases rapidly after lambing and falls to the basal level in the 4th postpartum week. The PAG level in sheep is influenced by the number of fetuses and fetal sex (18, 50). In cattle and sheep, PSBP is a pregnancy-associated glycoprotein identified as an essential compound released by the fetus to help maintain pregnancy. Its level rises gradually from the 3rd to the 9th week of pregnancy, then falls significantly higher than in singleton pregnancies (32). The disadvantages of the method include the high cost of progesterone analysis, the long process, the need for a laboratory, and the limited accuracy in separating singleton from multiple pregnancies. (41).

In sheep, pregnancy proteins (PAG and PSBP) are of placental origin (55) and can be present in the maternal circulation from the 3rd week of gestation until the 2-3rd week postpartum (31, 54). PAG, widely used in early pregnancy diagnostics, is used to detect embryonic/fetal death, identify twin pregnancies, and monitor placental functions (10, 25, 30). PAG is identified in many sheep breeds after the third or fourth week of pregnancy. Its level rises gradually from the 3rd to the 9th week of pregnancy, then rises again from the 17th to the lambing week, with variability between the 9th and 17th weeks. It decreases rapidly after lambing and falls to the basal level in the 4th postpartum week. The PAG level in sheep is influenced by the number of fetuses and fetal sex (18, 50). In cattle and sheep, PSBP is a pregnancy-associated glycoprotein identified as an essential compound released by the fetus to help maintain the corpus luteum (40). The binucleate cells of the fetal trophoectoderm release this protein (18).

This study aimed to determine the levels of pregnancy proteins (PAG and PSBP) to accurately predict singleton and twin pregnancies in Awassi sheep. It is also thought that these levels will be instrumental in complementing other methods of estimating fetal numbers. The study will be useful in determining the number of fetuses of Awassi ewes in the early stages of pregnancy to enable more effective pregnancy management.

Material and methods

This study was conducted with the approval of the Harran University Animal Experiments Local Ethics Committee dated 07/05/2021, number 2021/004.

Animal selection, oestrus synchronization, and mating.

On a private farm in the Eyyübiye District of Şanlıurfa province, a total of 40 Awassi breeds of sheep, aged 3 years to 5 years, were selected using a random sampling method under identical feeding and management conditions. Animals underwent real-time transabdominal ultrasonography of the genital organs (Hasvet 838®, Hasvet, Turkey), and this included animals in which no pathological problems such as hydrometra and pyometra were detected. Since the twin rate of Awassi sheep is low, a progesterone-based protocol was used in order to achieve this, a sponge impregnated with progesterone (30 mg progesterone acetate, Ovagen, Bioniche, Australia) was used. The vulva and perineal areas of the sheep included in the study were wiped with antiseptic water and dried. The vaginal sponge was then inserted into the vagina using a unique applicator (on day 0). On day 11, 2 ml of PGF2α (10 mg dinoprost tromethamine, Dinolytic, Pfizer) was injected intramuscularly. On day 12 of use, the vaginal sponge was removed, and 500 IU PMSG (Ovagen PMSG, Bioniche) was injected intramuscularly. For three days after the injection of PMSG, estrus monitoring was performed with a 30 minute search ram at 8 hour intervals, and estrus ewes were crossed with fertile rams.

Pregnancy examination, formation of experimental groups, and collection and analysis of blood samples.

The first pregnancy examination and determination of the number of fetuses in the sheep after rearing were performed on day 30, using real-time ultrasound both transrectally and transabdominally with a linear array probe at a frequency of 5 MHz. In this examination, the presence of hypochloic embryos and placenta in the offspring water were found in a non-echogenic region of the uterus, and were considered to be evidence of a positive pregnancy. The pregnant sheep were then separated into two groups based on the number of offspring they were carrying. The first group of the study (Group 1) consisted of single pregnant ewes (n = 20), while the second group (Group 2) consisted of twin pregnant sheep (n = 20). Blood samples were collected from the external jugular vein in 5 ml tubes containing a coagulation activator for PAG and PSBP evaluation. The fetal count was monitored with ultrasonography in both sheep study groups on day 30, 45, 60, 75 and 90 of gestation. The blood samples were transported to the laboratory under cold conditions, centrifuged at 3000 rpm for 10 minutes, and their samples of serum were collected and stored at −80°C until analysis. The levels of sheep pregnancy-specific protein B (PSBP Elisa kit, BT Lab) and sheep pregnancy-associated glycoprotein (PAGs Elisa kit, BT lab) in the sera were determined by the ELISA method using a commercial kit.

Statistical analysis.

Power was obtained by taking at least 80% (and Type-I error of 5%) for each variable in calculating the sample size of this study in order to determine the pregnancy protein (PAG and PSBP) levels in the estimation of singleton and twin pregnancies in Awassi sheep. Shapiro-Wilk (n < 50) and Skewness-Kurtosis tests were used to test whether the continuous measurements in the study were normally distributed. As the measurements were normally distributed, parametric tests were then applied. Descriptive statistics for continuous variables in the study mean standard deviation, minimum and maximum. Categorical variables were expressed as numbers and percentages. An independent-sample T-test was calculated to compare the measurements in the groups. ANOVA for Repeated Measurements was used to compare the measurements by time. The Bonferroni Post-Hoc Multiple Comparison Test was used to determine the times that made the difference after repeated ANOVA. Pearson correlation coefficients were calculated to determine the relationships between PAG and PSBP measurements. The statistical significance level (a) was set at 5% in the calculations, and the SPSS statistical package program (IBM SPSS for Windows, ver. 24) was used for the analysis.

Results and discussion

Intergroup comparison of pregnancy proteins.

Table 1 shows the results of intergroup comparisons of pregnancy proteins measured on the study groups’ specific days. All the PAG 30, PAG 45, PAG 60, PAG 75 and PAG 90 measurements showed a statistically significant difference between singleton and twin pregnant sheep (p < 0.05). In other words, measurements of PAG 30, PAG 45, PAG 60, PAG 75 and PAG 90 in twins were
all shown to be higher. In addition, all PSBP 30, PSBP 45, PSPB 60, PSBP 75 and PSBP 90 measurements revealed a statistically significant difference between singleton and twin pregnant sheep ($p < 0.05$). Therefore, the PSPB values measured on the measurement days were higher in twin pregnant ewes.

Comparison of pregnancy proteins according to intragroup time. Table 2 shows the comparison results of the pregnancy proteins in the study groups based on the time within the group. A statistically significant difference was found in singleton and twin pregnant ewes when using PAG measurement as a function of time within the group ($p < 0.05$). PAG values also differed on all days of measurement. In general, it was observed that the time-dependent PAG value also increased, while decreasing on day 90.

A statistically significant difference was found between times concerning PSBP measurement in singleton and twin pregnant ewes ($p < 0.05$) and PSBP values differed from each other on all days of measurement. In general, it was observed that the PSBP value showed a zigzag change, first increasing and then decreasing.

Intergroup pregnancy protein correlation analysis results. Table 3 shows the results of the intergroup pregnancy protein correlation analysis. A statistically significant correlation was found between PAG 30 and PSPB 30 levels in single pregnant ewes ($p < 0.05$). This relationship was found to be 45.2% positive and when the PAG 30 value increased, the PSPB 30 value also increased. Similarly, a statistically significant correlation was found between the dual measurements of PAG 45–PSBP 90, PAG 60–PSBP 90, PAG 75–PSPB 90 ($p < 0.05$). This correlation was found to have a negative direction. No statistically significant relationship was found between the other binary values except the above measurements in twin pregnant ewes ($p > 0.05$). A statistically significant correlation was found between the dual measurements of PAG 45–PSBP 30, PAG 45–PSBP 75, PAG75–PSPB 75, PAG 90–PSBP 30, and PAG 90–PSPB 75 in twin pregnant ewes ($p < 0.05$). No statistically significant relationship was found between the other binary values except the above measurements in twin pregnant ewes ($p > 0.05$).

### Table 1. Intergroup comparison results of pregnancy proteins (ng/ml)

| Proteins | Single pregnancy group | Twin pregnancy group |
|----------|------------------------|----------------------|
|          | Mean | St. Dev. | Min. | Max. | Mean | St. Dev. | Min. | Max. | *p  |
| PAG30    | 0.30 | 0.02    | 0.27 | 0.33 | 0.42 | 0.02    | 0.39 | 0.45 | 0.001 |
| PAG45    | 0.50 | 0.02    | 0.47 | 0.53 | 0.65 | 0.01    | 0.62 | 0.66 | 0.001 |
| PAG60    | 1.00 | 0.02    | 0.97 | 1.03 | 1.52 | 0.05    | 1.45 | 1.60 | 0.001 |
| PAG75    | 1.30 | 0.07    | 1.20 | 1.40 | 1.79 | 0.05    | 1.71 | 1.87 | 0.001 |
| PAG90    | 1.11 | 0.05    | 1.28 | 1.20 | 1.61 | 0.05    | 1.50 | 1.70 | 0.001 |
| PSPB30   | 38.27| 1.97    | 35.24| 41.54| 50.39| 1.90    | 47.36| 53.62| 0.001 |
| PSPB45   | 95.92| 6.53    | 85.24| 105.52| 130.88| 6.80   | 120.50| 140.98| 0.001 |
| PSPB60   | 73.15| 2.03    | 69.38| 75.98| 115.48| 3.20   | 110.68| 120.98| 0.001 |
| PSPB75   | 111.02| 1.74   | 107.65| 113.54| 142.67| 1.77   | 140.25| 145.97| 0.001 |
| PSPB90   | 104.72| 2.50   | 100.25| 108.65| 129.93| 3.00   | 125.98| 135.97| 0.001 |

*Explanation: * significance levels according to Independent T-test results

### Table 2. Comparison results of pregnancy proteins according to intragroup time (ng/ml)

| Proteins | Single pregnancy group | Twin pregnancy group |
|----------|------------------------|----------------------|
|          | Mean | St. Dev. | Min. | Max. | Mean | St. Dev. | Min. | Max. | *p  |
| PAG30    | 0.30a| 0.02    | 0.27 | 0.33 | 0.42a| 0.02    | 0.39 | 0.45 | 0.001 |
| PAG45    | 0.50b| 0.02    | 0.47 | 0.53 | 0.65b| 0.01    | 0.62 | 0.66 | 0.001 |
| PAG60    | 1.00c| 0.02    | 0.97 | 1.03 | 1.52c| 0.05    | 1.45 | 1.60 | 0.001 |
| PAG75    | 1.30d| 0.07    | 1.20 | 1.40 | 1.79d| 0.05    | 1.71 | 1.87 | 0.001 |
| PAG90    | 1.11e| 0.05    | 1.28 | 1.20 | 1.61e| 0.05    | 1.50 | 1.70 | 0.001 |
| PSPB30   | 38.27a| 1.97   | 35.24| 41.54| 50.39a| 1.90   | 47.36| 53.62| 0.001 |
| PSPB45   | 95.92b| 6.53   | 85.24| 105.52| 130.88b| 6.80  | 120.50| 140.98| 0.001 |
| PSPB60   | 73.15c| 2.03   | 69.38| 75.98| 115.48c| 3.20  | 110.68| 120.98| 0.001 |
| PSPB75   | 111.02d| 1.74  | 107.65| 113.54| 142.67d| 1.77  | 140.25| 145.97| 0.001 |
| PSPB90   | 104.72e| 2.50  | 100.25| 108.65| 129.93e| 3.00  | 125.98| 135.97| 0.001 |

*Explanation: * significance levels according to ANOVA Test results in repeated measurements; a, b, c, d, e – shows differences over time according to Bonferroni Post Hoc multiple comparison test

### Table 3. Intergroup pregnancy protein correlation analysis results

|          | Single pregnancy group | Twin pregnancy group |
|----------|------------------------|----------------------|
|          | PAG30 | PAG45 | PAG60 | PAG75 | PAG90 | PAG30 | PAG45 | PAG60 | PAG75 | PAG90 |
| PAG30    | 0.452*| 0.222 | -0.006| 0.266 | -0.316| 0.164 | -0.540*| 0.216 | -0.176| 0.725**|
| PAG45    | 0.017 | 0.174 | -0.124| 0.039 | -0.021| 0.318 | 0.414 | -0.156| 0.437| -0.145|
| PAG60    | -0.208| -0.170| 0.372 | -0.051| 0.027 | 0.183 | -0.161| -0.277| -0.076| -0.105|
| PAG75    | -0.219| -0.049| 0.003 | 0.389 | 0.011 | -0.021| -0.512*| 0.184 | -0.461*| 0.601**|
| PAG90    | -0.012| -0.488*| -0.448*| -0.582**| -0.266| 0.038 | -0.262| 0.213 | -0.142| 0.456*|

*Explanation: * *p < 0.05; ** p < 0.01; r – Pearson correlation coefficients
Precise diagnosis of pregnancy and the number of fetuses in sheep is essential to prevent the slaughter of pregnant and breeding animals and to ensure the normal birth weight of offspring through appropriate feed rations during pregnancy. In addition, it is economically advantageous to use herd management plans; for example identifying non-pregnant animals and re-breeding them during the season, feeding them only for wool yield, or or removing them completely from the herd (1, 12). Although the number of pregnancies and fetuses can be accurately diagnosed using ultrasonography, studies continue to result in the development of management methods that can be more easily applied to large herds. A number of studies have been conducted recently that look at pregnancy-related proteins or genes in the blood as an alternative to ultrasonography for pregnancy diagnosis. These studies found that detecting pregnancy-related glycoproteins, especially in cattle and sheep, is much more practical compared with gene expression studies and requires fewer laboratory procedures under field conditions. Researchers continue to conduct important studies on this topic (6, 20). In this study, by determining the levels of pregnancy proteins (PAG and PSBP) in Awassi sheep it was possible to accurately predict single and twin pregnancies. If the fetal numbers in the early pregnancy period are known, more effective pregnancy management of the sheep is possible.

Ultrasonography is an established technology for monitoring pregnancy and reproductive status in small ruminants (13). In addition to early pregnancy diagnosis, it allows for the detection of twin pregnancies, determination of fetal sex, determination of fetal age, determination of fetal viability, detection of embryonic deaths, and determination of uterine and ovarian pathologies (13, 14). It enables repeated examinations of the reproductive tract without affecting an animal’s reproductive physiology or negatively impacting the embryo/fetus (14, 42). However, when it comes to fetal counting, factors such as operator experience, probe type, animal housing/restraint options, body condition score and gestation length can greatly influence the efficiency and accuracy of the count (4, 44). On day 25 of pregnancy, single and multiple fetuses were determined via transrectally administered ultrasonography (7.5 MHz) (42). An experienced operator achieved a success rate of 99% between day 46 and 93 of gestation when determining single or multiple fetuses with transabdominal ultrasonography (53). In this study, pregnancy diagnoses and the number of fetuses were determined on day 30 by transrectal ultrasonography examination. Subsequent tests included blood sampling for PAG and PSBP measurements to determine if the pregnancy was healthy. As a result, fluctuations in pregnancy protein levels were prevented in the event of a potential embryonic death.

The ruminant placenta releases pregnancy proteins (PAG and PSBP), which enter the maternal circulation. As a result, the proteins can be considered as specific markers for the diagnosis of pregnancy. It is also indicated that, in addition to early pregnancy diagnosis, the proteins can be used to detect embryonic/fetal death, identify twin pregnancies, and monitor placental functions (3). It is noted that serum or milk PAG levels are influenced by many factors, such as milk yield (34), number of days into gestation (25), parity (34), race (27), twins (28), fetal sex, maternal weight (23), season (43), postpartum disease (26), and embryonic/fetal deaths (7). In this study, a sample was used using sheep of the same breed with similar maternal body condition scores, who gave birth during the same time period, and who had no postpartum problems. In other studies, it has been reported that in embryonic/fetal deaths occurring between day 28 and 120 of pregnancy, plasma and milk PAG levels (at approximately 4-5 weeks of pregnancy) are at lower levels than in animals whose pregnancy continues beyond the 5th week (30). In this study, the viability of the fetuses was checked using ultrasonography before each blood collection, and if no problems were detected, blood was collected.

Because plasma PAG levels may differ before day 26 of pregnancy, it has been determined that it is not a reliable indicator of pregnancy (8). PSBP in maternal circulation is often used for early pregnancy diagnosis because it is detected throughout pregnancy. However, Green et al. (11) reported two important negative aspects of using PSBP to diagnose pregnancy. The first is the risk of pregnancy not being diagnosed due to low circulating PSBP density in the first month of pregnancy and its variability. The second negative aspect is that PSBP remains in circulation for a long time after embryo death or birth due to its long half-life (8 days). Because of these two critical negatives, the use of other PAGs is more reliable for early pregnancy diagnosis (11). Early pregnancy diagnosis can be made with commercial PAG tests in dairy and beef cattle (35-38) using blood serum, milk, or whole blood samples (5-21). The accuracy of pregnancy tests that detect PAGs in blood or milk has been reported to vary from 92-100%, and the results are generally reliable (35, 45). In this study, blood sampling to determine pregnancy proteins was started on day 30 of gestation, therefore avoiding the differences observed in the preceding days. In addition, animals who experienced embryonic deaths in controls with routine ultrasonography examinations were not included in the groups.

PAG levels can also be measured in whole blood, serum, plasma, and milk (6, 46). In addition to the ELISA method, some researchers have studied PAG levels using the RIA method (6, 51). When diagnosing pregnancy, PAG expression should be determined with RIA between days 18 and 20. It has been reported that pregnancy can be diagnosed with high accuracy from days 6, 16 and 20. Researchers found that RIA provides earlier and more accurate results than ELISA in diagnosing pregnancy by looking at the PAG level in the early stage of pregnancy (15).

However, it should be noted that the RIA method has some disadvantages, such as the risk of contamination
with radioactive material and the need for a better-equipped laboratories (19). This study is compatible with existing research as it used the ELISA method to determine the PAG level by taking blood for measurement starting from day 30 of pregnancy. Studies have shown that the bovine PAG kit can be used for PAG measurement in sheep (39, 46). This is because PAG molecules released from trophoblast cells in the placenta of ruminant cattle and sheep have a similar molecular structure, and the cDNA encoding PAG has 86% nucleotide similarity (39). In this study, measurements in sheep were performed with the bovine PAG kit.

Early diagnosis of pregnancy is easier to detect with PAG, and it can be used as an alternative to clinical methods (32, 39). Although laboratory equipment is required for PAG detection of pregnancy, pregnancy diagnosis takes less time with PAG kits suitable for this method. Pregnancy diagnosis can also be performed under clinical/field conditions using visual inspection kits (19). This study reported that the mean PAGs value, which was 0.08 on day 21 of gestation and 0.21 on day 28 of gestation, reached 0.38 from day 35 of gestation. However, on day 42 of pregnancy, the mean value on day 35 declined slightly, although this decrease was not statistically significant. From day 42 of gestation, the average PAG value began to exceed the value on day 35, reaching 0.49 on day 49, 0.64 on day 56, 0.87 on day 63, and 0.97 on day 70, and the average PAG value increased continuously with advancing gestational age (15). A similar study in sheep reported that PAG decreased on day 49 and increased in the following days (46).

This study observed that the PAG level increased with time while also decreasing at day 90. The reason why there was no decrease in PAG level in the early period of our study is because the measurement interval was longer and did not coincide with these days. PAG measurement of singleton and twin pregnant ewes was carried out between days 18-49, and the PAG values of twin pregnant ewes were significantly higher on all days of measurement (6). In another study conducted in sheep, it was reported that plasma concentrations of PAG were significantly higher in multiple pregnancies on days 43-56 and days 76-87 of gestation, when compared with singleton pregnancies (17). In this study, PAG measurements in singleton pregnant ewes at days 30, 45, 60, 75 and 90 were 0.3, 0.5, 1.54, 1.30 and 1.11, and PAG measurements in twin pregnant ewes were 0.42, 0.65, 1.52, 1.79, 1.61. A statistically significant difference was observed both between groups and within groups as a function of time (p < 0.05). The measurement results were also higher in twin pregnant ewes than in singleton pregnant ewes on all the days the measurement was carried out. This corresponds with the data in literature. It has been reported that higher PAG concentrations in twins compared to singletons are probably due to a higher number of attachment sites and therefore higher contact areas between the fetal placenta and maternal caruncles (2, 32).

Some studies conducted to diagnose early pregnancy in cows and sheep reported that PSPB initially increased, then decreased, and then increased again (32, 47). This biphasic condition for pregnancy-associated glycoproteins is not observed by some researchers (36, 49). In this study, when both groups were examined on the measurement days, the biphasic condition showed an increase, a decrease, an increase, and a decrease, similar to the results in Steckeler et al. (46). PSPB measurement of singleton, twin, and triplet pregnancies belonging to different breeds was performed at 0-80 days of gestation, and the measurement was significantly higher in twin pregnancies between days 30 and 70, but no difference was observed in triplet pregnancies (33). A similar study in sheep found that PSPB levels were significantly lower in singleton gestations on days 48 and 85 of pregnancy when compared with twins and triplets. There was no difference in PSPB concentrations between twins and triplets (22). In another study to determine the number of fetuses in sheep, it was reported that PSPB concentration increased with the number of lambs when measured on day 46 of gestation. In the same study, PSPB concentration was found to be significantly higher in twin pregnant ewes than in single pregnant ewes, but although triple and quadruple pregnant ewes had higher PSPB concentrations, it was reported that there was no significant difference (29). In this study, PSBP measurement was determined as 38.27, 95.92, 73.15, 111.02 and 104.72 in singleton pregnant ewes at days 30, 45, 60, 75, and 90 and 50.39, 130.88, 115.48, 142.67 and 129.93 in twin pregnant ewes. A statistically significant difference was found both between groups and within groups as a function of time (p < 0.05). The measurement results were also higher in twin pregnant ewes than in single pregnant ewes on all the days the measurement was carried out, and this corresponds with the data in the literature.

In conclusion, using the ELISA method to measure PAG and PSPB in sheep’s blood is an accurate diagnostic tool for predicting singleton and twin pregnancies. A single blood sample taken during early pregnancy to determine the number of fetuses carried by a sheep would also allow breeders to make more informed herd management decisions.

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