Medit Vet J, 10(1): 458-464, 2025
DOI: 10.24880/meditvetj.1642231

Research Article

Influence of fetal sex and litter size on pregnancy-associated glycoprotein concentration at the end of the embryonic period in Hasak ewes

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Key Words:
ewe
fetal sex
litter size
PAG
pregnancy diagnosis

Received : 19 February 2025
Revised : 18 April 2024
Accepted : 20 April 2025
Published : 30 April 2025
Article Code : 1642231

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ABSTRACT

Reproductive efficiency is crucial for the economic profitability of sheep flocks and is strongly influenced by effective reproductive management. Accurate pregnancy diagnosis and fetal counts help breeders make informed decisions regarding the nutrition and care of pregnant ewes. This study aimed to determine whether plasma concentrations of pregnancy-associated glycoproteins (PAG) on day 35 of gestation can predict the number and sex of fetuses in Hasak ewes. Seventy-five pregnant Hasak ewes (2-7 years old), maintained under uniform conditions, were randomly selected. Pregnancy and the number of fetuses were determined on day 35 post-mating using transrectal ultrasound with a 7.5 MHz linear probe. Immediately after pregnancy diagnosis, blood samples were taken from the jugular vein of all selected ewes, and the plasma samples were separated. At lambing, the ewes were categorized into five groups according to the number and sex of lambs born: Ewes with a single male lamb (SM group, n=27), ewes with a single female lamb (SF group, n=27), ewes with twin male lambs (TM group, n=8), ewes with twin female lambs (TF group, n=7), ewes with one male and one female lamb (TMF group, n=6). Plasma PAG concentrations were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit. Differences in PAG concentrations between groups were analyzed using independent samples t-tests and one-way ANOVA. The results indicated that neither the number nor the sex of the fetuses significantly influenced the PAG concentrations in the plasma of Hasak ewes on day 35 of gestation ($p>0.05$). In conclusion, plasma PAG concentration on day 35 of pregnancy is not a reliable predictor of the number or sex of fetuses in Hasak ewes.

INTRODUCTION

Sheep are adaptable to diverse breeding conditions and have a relatively short gestation period. Their high prolificacy makes them suitable for various production goals, including meat, milk, and wool (Goldansaz et al., 2022). Due to these characteristics, sheep farming remains a significant industry within the global livestock sector, providing an indispensable contribution to human life with its diverse production outputs and the industrial products derived from them (Aydın et al., 2024). Flock-based sheep farming is often influenced by socio-economic factors, with profitability largely depending on production volume and efficiency (Simões et al., 2021). Effective herd management requires a balance between production and reproduction, with reproductive efficiency playing a central role in this process. In intensive sheep farming, reproductive efficiency is an important profitability factor, ensuring flock sustainability and influencing overall meat and milk yields (Theodoridis et al., 2018).

Reproductive efficiency refers to the number of lambs born per ewe exposed to a ram during a breeding season. Various factors such as breed, prolificacy, nutrition, conception rate, and viability of the embryo and fetus, influence this efficiency. As a result of reproductive flock management, reproductive efficiency varies considerably between sheep flocks. Reproductive flock management consists of various components, and determining the number of pregnant ewes and the number of lambs they carry as early as possible in a breeding season is shown as the most fundamental component for success. If pregnancy is detected as early as possible after mating during the breeding season, non-pregnant ewes can be rebred, removed from the flock, and unnecessary feed costs avoided, thereby increasing the pregnancy rate of the flock (Goldansaz et al., 2022). The main objectives in the management of pregnant ewes are to complete the pregnancy with a successful birth and to obtain live and healthy lambs with optimal birth and weaning weights (Fthenakis et al., 2012). During pregnancy, maternal nutrition is a crucial factor that directly affects fertility, lamb survival, and growth performance. Knowing the number of fetuses carried by pregnant ewes allows the implementation of a nutritional plan compatible with their nutritional requirements. Feeding pregnant ewes according to the number of fetuses they are carrying can help reduce the incidence of metabolic disorders such as pregnancy toxemia and hypocalcemia, as well as dystocia associated with high birth weight in singleton pregnancies. It can also help to optimize lamb birth weight, increase survival rates and reduce production costs (Jones and Reed, 2017; De Carolis et al., 2020). Furthermore, production costs can be reduced by ration optimi-

To cite this article:
Peker C, Ulutaş H, Akbulut NK, Kırbaş M, Uçar EH, Sari D, Atlı MO, Köse M. Influence of fetal sex and litter size on pregnancy-associated glycoprotein concentration at the end of the embryonic period in Hasak ewes. Medit Vet J. 2025; 10(1): 458-464.

sation according to the nutritional requirements of pregnant animals. In meat-breed sheep, multiple births are generally undesirable due to the lower birth weight of the lambs and the lower milk production capacity of the dams. Multiple births can lead to increased lamb mortality and negatively affect lamb meat production (Trabzon and Öztürk, 2019; Alataş, 2021). Predicting the number and sex of lambs of pregnant ewes in advance facilitates more accurate flock management decisions, such as flock renewal and flock expansion. It also enables the creation of markets for breeding animals based on the number and sex of lambs they carry and supports the development of future projections (Fthenakis et al., 2012). In summary, the development of a cost-effective, easy-to-use, and reliable method for determining the number of embryos/fetuses of pregnant ewes can significantly improve sheep health, lamb survival rates, animal welfare, and farm sustainability (Pickworth et al., 2020).

Under field conditions, transabdominal ultrasound is the most preferred technique for pregnancy diagnosis and determination of the number of fetuses in small ruminants, and it is generally recommended to be performed after 35 days of pregnancy (Barbagianni et al., 2017). However, the effectiveness of ultrasonography, especially in determining the number of fetuses, can be limited by several factors such as the experience of the operator, the type of probe used, the stage of pregnancy, and the facilities of the farm (availability of veterinarians, access to services, labor and time management) (Bretzlaff and Ramano, 2001; Sharkey et al., 2001). Gestation length is a genetically encoded reproductive trait in farm animals, and it is approximately 150 days in ewes (Jainudeen and Hafez, 2000). Most offspring losses during pregnancy occur in the embryonic period, when there is a delicate balance and reciprocal interactions between the embryo and the dam in the establishment and maintenance of pregnancy, especially prior to implantation. The rate of pregnancy loss decreases significantly as the conceptus develops and grows (Rickard et al., 2017; Chundekkad et al., 2020). Considering that pregnancy losses after day 30 of pregnancy are only 1-5%, it can be concluded that the pregnancy will most likely continue after the embryonic period is completed (O'Connell et al., 2016; Rickard et al., 2017). All these data make the 35th day of pregnancy, which is the end of the embryonic period, an important reason for preference in terms of pregnancy diagnosis, health evaluation, and determination of the number of fetuses in ewes (Tekin and Köse, 2022). This situation provides initial data for the development of alternative methods to ultrasonography.

Currently, pregnancy-associated glycoproteins (PAG) are among the primary biomarkers investigated for pregnancy diagnosis, monitoring pregnancy health, and determining the litter size in ruminants (Barbato et al., 2022). The main source of these molecules is binuclear trophoblast cells that migrate during the implantation process, connect to the epithelial cells of the endometrium, and are involved in the formation of cellular plaques called syncytium. Pregnancy-associated glycoproteins are products of gene homologs that are well conserved throughout evolution in species of the Cetartiodactyla order, including cows and sheep, and can be detected in maternal peripheral blood from the 3rd week of pregnancy in ewes (Haugejorden et al., 2006). Studies have confirmed that PAGs are molecules secreted by trophoblast cells and have shown that their concentration in maternal blood increases with the increase in functional placental mass as pregnancy progresses. This increase is particularly marked in early pregnancy (Uçar et al., 2018; Barbato et al., 2022). Indeed, the detection of PAG using commercially available enzyme-linked immunosorbent assay (ELISA) kits enhances their applicability under field conditions and highlights their potential as reliable diagnostic tools in veterinary practice (Friedrich and Holtz, 2010; de Miranda et al., 2017). In addition to the usefulness of these molecules for pregnancy diagnosis, it is also emphasized that they may play crucial roles in the regulation of the maternal immune system during early pregnancy, embryo implantation, and blastogenesis, which are essential for pregnancy maintenance and health (Pohler et al., 2013; Pohler et al., 2016). In ruminants, the decrease or lack of differentiation and development of binuclear cells has been shown to be a potential factor leading to pregnancy loss (Reese et al., 2019; Barbato et al., 2022; Wooding, 2022). In addition, studies have summarized that in ewes, which have a better capacity for multiple births than other species, peripheral blood PAG concentrations are influenced by several breeding-related factors, including breed, litter size, fetal sex, and gestational age (Haugejorden et al., 2006).

The Hasak sheep is a meat breed developed through crossbreeding studies using Akkaraman as the maternal line and Hampshire and German Blackheaded Mutton sheep as the paternal lines (Köse et al., 2012). Several studies have been conducted on the anatomical characteristics, blood parameters, and the growth and fattening performance of lambs of this breed (Tekin et al., 2005; Şimşek et al., 2015; Teke et al., 2017). However, the reproductive traits of this breed, which are crucial for the continuity and spread of the breed, have not been sufficiently researched. Limited studies on the fertility of this breed have found that reproductive performance indicators are not as expected, with low lambing rates per ewe and a low twin rate (Köse et al., 2012; Trabzon and Öztürk, 2019). The aim of this study was to contribute to the knowledge of reproductive physiology by determining plasma PAG concentrations on day 35 of pregnancy in Hasak ewes carrying male or female single or twin fetuses and evaluating the effects of the number and sex of fetuses on PAG concentrations during pregnancy.

MATERIALS and METHODS

Animals

The study was conducted on pregnant Hasak ewes kept in the Small Ruminant Breeding Department of the Bahri Dağdaş International Agricultural Research Institute located in Karatay/Konya/Turkey. All animals included in the study were clinically healthy. The animals were 2 to 7 years old, with body condition scores (BCS) ranging from 2.50 to 4.00 on a 1-5 scale (Russel et al., 1969).

Housing and feeding system

All ewes were housed under uniform conditions in a se-

mi-open barn and fed a balanced ration prepared at the institute's feed unit, following NRC (2007) guidelines. The animals had ad libitum access to water. All routine health checks, vaccinations, and parasite treatments were carried out regularly before the breeding season.

Estrus detection, breeding, and pregnancy diagnosis

During the breeding season, estrus was detected in ewes by mating acceptance behavior with teaser rams. Ewes exhibiting estrus were mated with fertile, same-breed rams that had previously passed fertility testing. The details of the mating were recorded and the day of mating was considered day 0 in the study. On day 35 after mating, pregnancy examinations were performed via a transrectal approach using a B-mode real-time ultrasound device (Scanner 480 Vet, Esaote Pie Medical, Maastricht, The Netherlands) equipped with a 7.5 MHz probe. Among the ewes whose pregnancy was diagnosed by observing the embryonic heartbeat, 75 Hasak ewes were randomly selected using a blind sampling method and included in the study.

Blood collection and plasma samples

Immediately after pregnancy diagnosis (on day 35 of pregnancy), approximately 10 mL of venous blood was collected from the jugular vein of the 75 ewes included in the study in Na-EDTA-containing vacuum tubes (BD Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA). To separate the plasma, the collected samples were centrifuged at 3000 rpm for 20 minutes without delay. The plasma samples were aspirated using an automatic pipette and transferred to Eppendorf tubes. The prepared samples were immediately frozen and stored at -20°C until the PAG analysis was performed.

Experimental design

Nutritional and health monitoring of the ewes in the study was carried out regularly from the 35th day of pregnancy until the postpartum period. The lambing records of the ewes were examined and information including the lambing date, type of birth, and number and sex of lambs were obtained from the flock birth register. The results of the ultrasonographic pregnancy diagnosis performed on the 35th day of pregnancy were confirmed. Based on these data, the following five groups were formed: Ewes with a single male lamb (SM group, n=27), ewes with a single female lamb (SF group, n=27), ewes with twin male lambs (TM group, n=8), ewes with twin female lambs (TF group, n=7), ewes with one male and one female lamb (TMF group, n=6).

Determination of plasma PAG levels

The plasma PAG concentrations were determined by analysis at the Research Laboratory of the Dicle University, Faculty of Veterinary Medicine, Department of Obstetrics and Gynecology, and the Laboratory of the Dicle University Health Sciences Application and Research Center. The plasma PAG concentrations were measured using a commercial ELISA kit (Bovine Pregnancy-Associated Glycoproteins ELISA Kit, BT LAB, Zhejiang, China). The measurement procedures were performed according to the manufacturer's instructions. The manufacturer reported the kit's sensitivity as 0.1 µg/mL, the standard curve range as 0.05–20 ng/mL, the intra-assay coefficient of variation as <8%, and the inter-assay coefficient of variation as <10%.

Statistical analysis

The statistical analysis of the study was performed using SPSS 24.0 package program (SPSS, IBM SPSS Statistics, Chicago, IL, USA). Data were presented as mean ± SEM and $p<0.05$ was considered statistically significant. The distributions of the data were tested using the Shapiro-Wilk test. The analysis of the data concerning the groups was performed using the one-way ANOVA test. The mean values of the groups were compared using the independent t-test.

RESULTS

Table 1 shows the mean plasma PAG concentration, total lamb weight, mean lamb weight, and gestation length for the groups in the study. No statistically significant differences were found between the groups in terms of plasma PAG concentration and gestation length ($p>0.05$). The mean plasma PAG concentration was also similar in ewes with a single lamb and twin lambs, regardless of the sex of the lambs ($p>0.05$). However, total lamb weight was significantly higher in the TM group than in the other groups, and it was also higher in the TF and TMF groups than in the SM and SF groups ($p<0.05$). In addition, the average weight of lambs was significantly higher in the SM group than in the TF and TMF groups, while it was higher only in the SF group than in the TMF group ($p<0.05$).

Table 1. The relationship between litter size and sex at day 35 of pregnancy with plasma PAG levels, lamb birth weight, and gestation length in Hasak ewes.

Groups (n)	PAG-I (ng/mL)	PAG-II (ng/mL)	Total lamb weight (kg)	Average lamb weight (kg)	Gestation length (day)
SM (27)	3.20±0.37	3.38±0.26	4.95±0.20^{C}	4.95±0.20^{A}	148.89±3.46
SF (27)	3.55±0.37		4.70±0.16^{C}	4.70±0.16^{AB}	148.96±2.10
TM (8)	4.01±0.67		9.10±0.42^{A}	4.55±0.21^{ABC}	149.88±1.36
TF (7)	2.78±0.72	3.24±0.42	7.78±0.58^{B}	3.89±0.29^{C}	148.86±1.77
TMF (6)	2.76±0.78		7.94±0.61^{B}	3.97±0.30^{BC}	148.67±2.80

SM: Ewes with a single male lamb, SF: Ewes with a single female lamb, TM: Ewes with twin male lambs, TF: Ewes with twin female lambs, TMF: Ewes with one male and one female lamb, PAG-I: PAG concentration based on the number of lambs and sex, PAG-II: PAG concentration based on the number of lambs, A, B, C: Different superscripts in the same row indicate a significant difference between the columns at the $p<0.05$ level.

DISCUSSION

In sheep farming, breeders generally prefer breeds with high lamb yield per ewe. Within sheep breeds, certain breeds such as Sakiz and Romanov are particularly known for their high prolificacy. However, in meat-breed sheep, the increased numbers of fetuses during pregnancy can lead to nutritional deficiencies in the dams, especially during the late gestation period and early neonatal period due to the rapid fetal growth. This nutritional imbalance contributes to reduced lamb viability and higher neonatal lamb mortality, making multiple births an undesirable trait in some meat breed sheep. The Hasak sheep is also a meat-breed sheep. In a previous study, it was found that the number of lambs born per ewe in Hasak ewes was 113%, the survival rate of lambs at day 90 was 86.7% and the survival rate of twin lambs at day 90 was significantly lower compared to singleton lambs. It was also found that the effect of birth type on survival rate could be related to the pregnancy of the ewe, its ability to care for the lamb after birth, and its ability to produce sufficient milk, as well as the birth weight of the lamb (Trabzon and Öztürk, 2019). Therefore, estimating the number of fetuses during pregnancy is of great importance in meat sheep production, both to ensure healthy lamb births and to increase lamb meat production. In this study, it was aimed to investigate the effect of litter size (singleton or twin births) and the sex of fetuses on maternal circulating PAG concentrations on day 35 of gestation, which marks the end of the embryonic period, in Hasak ewes.

The ability to predict ewes with multiple fetuses in a flock enables the implementation of better management strategies and the maximization of productivity while improving animal welfare (Llanes et al., 2019). Currently, ultrasonography is the most commonly used method for both pregnancy diagnosis and litter size determination in small ruminant reproductive management. However, it is well known that ultrasound-based pregnancy diagnosis and especially litter size determination are subject to various limitations (Bretzlaff and Romano, 2001; Sharkey et al., 2001). Therefore, performing laboratory analysis to determine PAG concentrations in blood and body secretions, especially during early pregnancy, could open up new applications not only for pregnancy diagnosis but also for litter size determination. This approach could offer breeders an alternative to ultrasound examination and thus enable more informed management decisions (Llanes et al., 2019).

Studies have been conducted to determine litter size during pregnancy by measuring PAG concentrations using radioimmunoassay (RIA) or ELISA techniques in various ruminant species and breeds, including cattle, buffalo, goats, and sheep. In studies in ewes, Karen et al. (2006) reported that PAG concentrations were higher in ewes pregnant with twin or multiple fetuses than in ewes pregnant with a single fetus when measured on days 43-56 of pregnancy using a homologous RIA assay. Similarly, Pickworth et al. (2020) and Çebi & Akköse (2024) found increased PAG concentrations in ewes carrying multiple fetuses when measured using ELISA on days 46 and week 7 of pregnancy, respectively. Barbato et al. (2009) and El Amiri et al. (2015) reported significant differences in PAG concentrations between ewes carrying one lamb and those carrying multiple lambs as early as day 18 of pregnancy using a homologous RIA test. However, it has been noted that this difference may not be clearly detectable until day 28, depending on the specificity of the antisera used (El Amiri et al., 2015). These results suggest that the RIA technique could be used to discriminate between singleton and multiple pregnancies in ewes based on PAG levels during early gestation. However, it has been emphasized that differences in the sensitivity of antisera to different epitopes may lead to discrepancies in the results obtained using this method (El Amiri et al., 2015). Most of these mentioned studies focused on the comparison of PAG concentrations between ewes pregnant with one and multiple fetuses (Karen et al., 2006; Barbato et al., 2009; El Amiri et al., 2015). Studies conducted on ewes pregnant with single and twin lambs suggest that the differences in PAG concentrations only become apparent at later stages of pregnancy (Ledezma-Torres et al., 2006; Pickworth et al., 2020; Çebi and Köse, 2024). In a study using a heterologous RIA test, differences in PAG concentration between ewes carrying single and twin lambs were found to be detecteble at 8-9 weeks of pregnancy (Ledezma-Torres et al., 2006). In two other studies, significant differences in PAG concentration were found in ewes with single or twin lambs at 21 weeks of pregnancy (Ranilla et al., 1997) and shortly before parturition (De Carolis et al., 2020). However, studies using the ELISA technique indicate that the difference in PAG concentrations between singleton and twin pregnant ewes becomes more pronounced after the completion of the embryonic period (Alkan et al., 2020; Pickworth et al., 2020; Çebi and Köse, 2024). Moreover, a recent study in ewes reported that PAG levels in animals carrying twin lambs, compared to those carrying a single lamb, decreased from day 30 of pregnancy onward (Akkuş and Yaprakçı, 2022). Similarly, studies in goats have shown that after the completion of the embryonic period, PAG concentrations in goats carrying a single kid, compared to those carrying multiple kids, decreased on days 45 (Singh et al., 2019), 48 (Llanes et al., 2019), and 85 (Lü et al., 2021) of pregnancy. Szelényi et al. (2015) also reported that bovine pregnancy-associated glycoprotein-1 (bPAG-1) concentrations on days 30 and 60 of pregnancy were similar between singleton and twin pregnant cows. They also found that the detection of twin pregnancies using bPAG-1 measurements was only possible from day 85 of pregnancy. When all these results are evaluated, it becomes clear that determining the number of fetuses in multiple pregnancies in the early stages of gestation remains a major challenge. Also in our current study, ELISA-based analysis at the end of the embryonic period showed no significant difference in PAG concentrations between singleton and twin pregnant ewes. Our results are consistent with most studies in the literature and suggest that measurement of PAG concentration in a single blood sample taken on day 35 of pregnancy is not sufficient to discriminate between ewes with single and twin fetuses. The inability to determine litter size by determining PAG concentrations at the end of the embryonic period in Hasak ewes and the resulting limitation in preventing twin pregnancies emphasize the need to find alternative indicators to ultrasonography. It is also recommended that new management strategies be developed, and preventive measures be taken to increase the survival rate of twin lambs that have lower

survival rates at weaning.

Diagnosis of fetal sex during pregnancy in ruminants serves several purposes and has implications for livestock production, including sheep production. The sex of the fetus is determined chromosomally at the beginning of pregnancy, at the same time as fertilization. However, under field conditions, the transition to the fetal stage must be completed to determine the sex of pregnant ewes. Ultrasound-based sexing, which relies on the identification of the genital tubercle, can only be performed after day 60 of gestation and requires an experienced clinician, making it a labor-intensive and time-consuming procedure (Barbagianni et al., 2017). Studies on methods of sex determination based on PAG concentration in maternal blood have not yielded successful results (Ledezma-Torres et al., 2006; Alkan et al., 2020). Also in our current study, plasma PAG concentrations at day 35 of gestation did not show significant differences concerning fetal sex. However, some studies suggest that fetal sex may influence PAG concentrations as pregnancy progresses. Ranilla et al. (1994) reported that maternal PAG concentrations in the blood of ewes with male fetuses increased from 19 weeks gestation. Similarly, De Carolis et al. (2020) suggested that fetal sex may influence PAG concentrations. They suggested that the increased PAG concentrations observed in ewes with male fetuses could be due to the higher weight of male fetuses in utero as gestation progressed. These results suggest that hormonal or placental factors related to fetal development may contribute to the variation in PAG levels in the later stages of pregnancy. Further studies examining the later stages of pregnancy could help clarify the possible relationship between fetal sex and PAG dynamics.

CONCLUSION

In conclusion, it was found that measuring plasma PAG concentration at the end of the embryonic period is not a reliable method for predicting the number and sex of fetuses in Hasak ewes, a meat breed. Similarly designed further studies conducted at advanced stages of pregnancy may be beneficial in assessing the effects of litter size and sex on maternal PAG concentrations.

DECLARATIONS

Ethics Approval

The study was conducted with the approval of the Animal Experiments Local Ethics Committee of Bahri Dagdas International Agricultural Research Institute (25.01.2023/153).

Conflict of Interest

The authors declare that there are no conflicts of interest for this study.

Consent for Publication

Not applicable.

Author contribution

Idea, concept and design: CP, HU, MK[5], NKA, MK[4]

Data collection and analysis: HU, NKA, DS, MK[4]

Drafting of the manuscript: CP, EHU, MOA, DS

Critical review: MK[5], MOA, EHU, DS

Data Availability

The data of this study are available from the corresponding author upon reasonable request.

Funding statement

The authors receive no financial support for this article.

Acknowledgements

We would like to thank the Bahri Dagdas International Agricultural Research Institute and its staff for providing animal material and their contributions to the field study.

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