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Plasma profile of Pregnancy Associated Glycoproteins during Postpartum period in Konya Merino ewes lambing single and twin

Perfil Plasmático de Glicoproteínas asociadas a la preñez durante el período posparto en ovejas Merino Konya con partos simples o dobles

***Eyyup Hakan Ucar*[1*], *Mehmet Kose*[2], *Mesut Kirbas*[3], *Faruk Bozkaya*[4], *Mehmet Osman Atli*[5]**

[1]*Aydin Adnan Menderes University, Faculty of Veterinary Medicine, Department of Obstetrics and Gynecology. Aydin, Turkey.*
[2]*Dicle University, Faculty of Veterinary Medicine, Department of Obstetrics and Gynecology. Diyarbakir, Turkey.*
[3]*Bahri Dağdaş International Agricultural Research Institute. Konya, Turkey.*
[4]*Harran University, Faculty of Veterinary Medicine, Department of Animal Science and Animal Nutrition/Department of Veterinary Genetics. Sanliurfa, Turkey.*
[5]*Harran University, Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemination. Sanliurfa, Turkey.*
**Corresponding author: hakan.ucar@adu.edu.tr*

ABSTRACT

This study investigated the plasma profile of Pregnancy Associated Glycoproteins (PAGs) after lambing in Konya Merino ewes using specific cattle pregnancy test kit. A total of 16 Konya Merino ewes were used as a material. Four groups were set up, ewes birthing a male lamb (SM group, n=4), a female lamb (SF group, n=4), twin male lambs (TM group, n=4) or twin female lambs (TF group, n=4). Blood plasma samples were taken on days 0 (lambing day), 3, 7, 10, 14 and then weekly until day 35. All samples were analysed using a bovine pregnancy test kit to detect of PAGs level. It was determined that there was a strong negative correlation between PAGs levels in peripheral blood and days after lambing (r^2=0.969; P<0.01). However, no relationship was found between PAGs level and lamb gender or birth type. In conclusion, plasma PAGs level decreases rapidly in Konya Merino ewes regardless of lamb gender and birth type. Moreover, the results showed that the plasma profile of PAGs after lambing in Konya Merino ewes can be monitored with a bovine commercial ELISA-based pregnancy test kit, and the test results can be used in decisions and assessments based on the levels of PAG molecules.

Key words: Konya Merino; Pregnancy Associated Glycoproteins; postpartum period

RESUMEN

En este estudio, se investigó el Perfil Plasmático de las Glicoproteínas asociadas a la preñez (PAGs) después del parto en ovejas Konya Merino utilizando un kit de prueba de preñez utilizado en el ganado vacuno. Se utilizó un total de 16 ovejas Konya Merino como material. Se establecieron cuatro grupos de ovejas que parieron a un cordero macho (grupo SM, n=4), una cordera hembra (grupo SF, n=4), corderos mellizos machos (grupo TM, n=4) o corderas mellizas hembras (grupo TF, n=4). Se tomaron muestras de plasma sanguíneo en los días 0 (día del parto), 3; 7; 10; 14 y luego semanalmente hasta el día 35. Todas las muestras se analizaron utilizando un kit de prueba de preñez bovino para detectar el nivel de PAGs. Se determinó que existe una fuerte correlación negativa entre el nivel de PAGs en la sangre periférica y los días después del parto (r^2=0,969; P<0,01). Sin embargo, no se encontró ninguna relación entre el nivel de PAGs y el género del cordero o el tipo de parto. En conclusión, el nivel plasmático de PAGs disminuyó rápidamente en ovejas Konya Merino independientemente del género y tipo de parto del cordero. Además, los resultados mostraron que el perfil plasmático de PAGs después del parto en ovejas Konya Merino se puede monitorear con un kit comercial de prueba de preñez bovino basado en ELISA, y los resultados de la prueba se pueden utilizar en decisiones y evaluaciones basadas en los niveles de las moléculas de PAGs.

Palabras clave: Konya Merino; glicoproteínas asociadas a la preñez; período postparto

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INTRODUCTİON

The placenta is a temporary and highly specialised tissue formed during pregnancy in mammals and removed from the maternal organism following the offspring delivery [1]. The placenta type of sheep (*Ovis aries*) is histologically defined as synepiteliochorial cotyledonata [2]. The fetal cellular composition of this type of placenta consists of mononuclear trophoblastic cells and binuclear trophoblastic cells. Binuclear trophoblastic cells migrate to the uterine endometrium during implantation and merge with epithelial cells forming multinucleated giant cells and syncytial plaques. Those cells synthesise and secrete various components such as Pregnancy Associated Glycoproteins (PAGs), progesterone, placental lactogen, growth factors, enzymes, and proteins. These components make important contributions to establishing and maintaining pregnancy via their local and systemic effects on both maternal and fetal sides [3, 4].

Pregnancy Associated Glycoproteins are placental antigens encoded by the gene family included in the aspartic proteinase family, formed by duplication and expansion of the pepsinogen F gene [5]. At the beginning of embryonic implantation, PAGs are expressed locally [6, 7, 8]. However, with the progression of implantation and placentation, they reach a much higher concentration than can be detected in maternal circulation on day 35. Moreover, the PAGs level in the maternal blood reaches the highest level at the end of the pregnancy due to the increase in the functional mass of the placenta [9, 10, 11]. It is stated that the migration of binuclear trophoblastic cells to maternal tissue and their association with endometrial cells further facilitate the detection and passage of PAGs into the maternal circulation [3]. Various studies have shown that the concentration of PAGs in maternal blood circulation can change under the influence of many factors such as species, race, number of fetuses, fetal sex, birth weight, and feeding, among others [9, 11].

The concentration of PAGs in the maternal blood reaches the highest level at the close period to birth, along with the development of fetal tissues and systems. Therefore, studying the transmission of PAGs to the offspring through the placental circulation and to colostrum would provide valuable information for their functional effects on pregnancy health and offspring health [9, 12]. For example, Hooshmandabbasi *et al.* [13] suggested that PAGs might be causally involved in developing retained fetal membranes by suppressing immunological processes in cows (*Bos taurus*). Moreover, still early, Dosogne *et al.* [14] suggested bPAG may be associated with the inhibition of the polymorphonuclear neutrophil leukocytes function of dairy cows during the early postpartum period. Compared to cow studies, few studies monitoring the PAGs levels in maternal plasma during pregnancy and postpartum period have been reported in sheep. In farm animals, identifying new markers for examination and evaluation of postpartum physiology, which is the preparatory stage of a new pregnancy after birth, is important for understanding the pregnancy process and making breed-specific breeding decisions [9, 11, 15]. In this study, it was aimed to determine the profile of PAGs during the postpartum period in the plasma of Konya Merino ewes that delivered single or twin lambs of different genders to contribute to the basic knowledge of the reproductive physiology and raising breeding of this breed, which is very well adapted to the conditions of Central Anatolia Region and is widely bred by sheep breeders in this geography.

MATERIAL AND METHODS

Animal material

The study was carried out on 16 Konya Merino ewes selected from the breeding flock that has grown at the Bahri Dağdaş International Agricultural Research Institute. Ewes, 3–5 years old, with a body condition score of 3–4 (based on a score 0 to 5), had given birth at least once before. Ewes not suffering from dystocia and retained placenta and did not have any health problems during the prepartum period or at the time of blood sampling were included in the study.

The ewes were grazed during the daytime on the pastureland of the Institute whenever the grazing conditions were appropriate. At night, they were housed in semi-open barns. The ewes were fed with an additional 2,500 ME and 15% crude protein concentrate feed, at a rate of 500 g per ewe per day, in addition to pasture grazing, from approximately three weeks before the start of the breeding season to the third week after the end of the breeding season. Until 6 weeks before lambing after the completion of the breeding season, the ewes were fed with roughage consisting of 1 kg legume hay and 1 kg wheat straw (*Triticum aestivum*) in addition to 2,250 ME and 13% crude protein concentrate feed at a rate of 400 g per ewe daily. At the period from about 6 weeks before the start of the lambing season until the completion of the study, the ewes were fed with sheep milk feed containing 2,400 ME and 14% crude protein (at a rate of 1500 g per sheep per day), as well as 1 kg of dry clover hay and 1 kg of wheat straw per sheep per day. Drinking water was provided as an *ad libitum.*

The ewes were kept with their lambs in the lambing area for the first 3 days after birth. At the end of this period, the lambs were separated from dams and allowed to suckle once a day until they were weaned. Milk was not collected from the ewes.

Collecting plasma samples from sheep

Four groups of ewes were formed based on the type of delivery as a single male lamb (SM, n=4), a single female lamb (SF, n=4), twin male lambs (TM, n=4), and twin female lambs (TF, n=4). The first blood sample was taken as soon as possible after lambs received colostrum on the day of birth (0^{th} day). The other blood samples in the study were taken on days 3, 7, 10, 14, 21, 28, and 35 with reference to the day of birth (0^{th} day). Blood samples were collected from the jugular vein of the ewes using 10 mL vacuum dipotassium ethylenediaminetetraacetic (K_2EDTA) tubes (10 mL, BD Vacutainer®, BD, Belliver Industrial Estate, Plymouth, UK) containing 18 mg of EDTA. All blood samples were centrifuged (M 4808 PR, Elektromag, Istanbul, Turkey) at 1,562 g·20 min^{-1} immediately. Plasma samples collected using an automatic pipette (Eppendorf Research Plus, Hamburg, Germany) were transferred to sample tubes. Tubes were stored at –80°C (MDF-U 3286S Sanyo, Japan) in the Research Laboratory of The Department of Veterinary Obstetrics and Gynecology at Dicle University until analysis.

The laboratory analyses of plasma samples

The concentration of PAGs in plasma samples was semi-quantitatively determined with a commercial kit using an enzyme-linked immunosorbent assay method (IDEXX Bovine Pregnancy test kit, Switzerland AG, Stationsstrasse 12, 3097 Liebefeld-Bern, Switzerland). The laboratory analyses were performed according to the manufacturer's instructions in the kit's manual. At the beginning of the analyses, the kit components and the plasma samples were kept at room temperature until their temperatures reached ambient

temperature without exposure to daylight. For the analyses, 25 µL of sample diluent was first added to the microplate wells. Then, for analysis control, 100 µL of control solutions were added to four wells, as two positive controls and two negative controls, from the standard kit solutions. The remaining wells were filled with 100 µL of plasma sample from each sample using a separate pipette tip for each plasma sample, according to the previously prepared sample pattern. The microplate covered with a parafilm was gently shaken and then incubated in a 37°C incubator (Nuve EN 120, Ankara, Turkey) for 60 min.

Subsequently, the plate was washed 5 times with washing solution (Flexiwash, Asys Hitech, Eugendorf, Austria) using an automatic 8-channel plate washer (Flexiwash, Asys Hitech, Eugendorf, Austria). Then, a 100 µL of detector solution (anti-PAG antibody) was added to each well, and the plate was covered with a parafilm and incubated at room temperature for an additional 30 min. Afterwards, the plate was washed 5 times in the microplate washer, and the detector solution was removed. Immediately after this, 100 µL of conjugate solution (anti-IgG-horseradish) was added to each well, and the plate was covered with an opaque material and kept at room temperature for 30 min. Then, the conjugate solution was also removed from the plate by washing the plate 5 times with the automatic plate washer, and 100 µL of tetramethylbenzidine substrate was added to each well, then the plate was kept at room temperature for 15 min. Afterwards, 100 µL of stop solution was added to each well to stop the reaction, and without delay, the absorbance value of each well at 450 nm was determined using a microplate titer spectrophotometer (Thermo Scientific, Multiskan GO, USA). In statistical analysis, the value obtained by subtracting the mean optical density value of the negative controls from the optical density value of each plasma sample was used as the optical density value of plasma samples.

Statistical analyses

To evaluate the effect of lamb gender (male-female) or type of birth (single birth-twin birth) on plasma PAGs-OD (optimal density) values during postpartum days, two-way analysis of variance with repeated measures was used. A paired sample t-test was used to compare the mean PAGs-OD values of the postpartum days. A Pearson's correlation analysis was performed to evaluate the relationship between total birth weight and PAGs-OD values within each postpartum days (0, 3, 7, 10, 14, 21, 28 and 35) and the mean PAGs-OD values on those days.

RESULTS AND DISCUSSİON

The mean total birth weight of single females, single males, twin females, and twin males were 4.00 ± 0.65, 4.05 ± 0.38, 6.80 ± 0.49, 7.00 ± 0.51.

A statistically significant effect of sampling time on the PAG-OD values was observed. This study's mean PAG-OD value rapidly decreased after delivery, falling below 0.3 on the 28th postpartum day. In the postpartum decreasing profile in the mean PAG-OD value, the first significant low value compared to the value at the delivery day was detected on the 10th postpartum day. The effect of lamb gender on PAG-OD values during postpartum days was insignificant for single and twin births (TABLE I).

When the PAG-OD values of ewes that gave birth to twins and those that gave birth to singles were compared without grouping them by sex, it was found that PAG-OD values were similar from the day of lambing up to day 21, on the other hand, on days 28 and 35, the PAG-OD value was higher in ewes that gave birth to twins compared to those that gave birth to singles. The values for these measurements are given in TABLE II, and the graph is shown in FIG. 1.

TABLE I
The change of plasma OD values of pregnancy-associated glycoproteins in the postpartum period in Konya Merino ewes with single and twin lambing according to the gender of the offspring

Postpartum Day	Single lambing		Twin lambing		General
	Female	Male	Female	Male	
0	1.12 ± 0.17	0.75 ± 0.17	1.21 ± 0.17	0.94 ± 0.17	1.00 ± 0.08 [a]
3	1.03 ± 0.16	0.69 ± 0.16	1.06 ± 0.16	0.99 ± 0.16	0.94 ± 0.11 [ab]
7	0,83 ± 0.19	0.79 ± 0.19	1.14 ± 0.19	0.99 ± 0.19	0.94 ± 0.09 [ab]
10	0.84 ± 0.23	0.54 ± 0.23	1.02 ± 0.23	0.89 ± 0.23	0.82 ± 0.11 [b]
14	0.46 ± 0.15	0.43 ± 0.15	0.69 ± 0.15	0.66 ± 0.15	0.56 ± 0.07 [c]
21	0.34 ± 0.11	0.23 ± 0.11	0.49 ± 0.11	0.41 ± 0.11	0.37 ± 0.05 [d]
28	0.11 ± 0.04	0.09 ± 0.04	0.21 ± 0.04	0.17±04	0.15 ± 0.02 [e]
35	0.07 ± 0.03	0.06 ± 0.03	0.12 ± 0.03	0.15 ± 0.03	0.099 ± 0.01 [f]
r	0.957**	0.963**	0.932**	0.967**	0.969**
The effect of postpartum day			*P*<0.01		
The effect of lamb gender			Not significant		
The effect of birth type			Not significant		

[a, b, c, d, e, f]: Different letters on postpartum days, show significant differences between postpartum days in the same column. *r*: Correlation coefficient between postpartum days and mean PAG levels in each group. **:Importance of correlation coefficient (*P*<0.01), Not significant: *P*>0.05

No statistically significant effect of lambing type (singlets or twins) or gender (male or female) was detected. No interaction between sampling time, gender or lambing type was observed.

On the other hand, significantly high correlation coefficients were observed between total birth weights and PAG-OD values on sampling days of 0, 7, 10, 21, 28 and 38.

Since PAGs are placenta-derived, scientific studies on these antigens in farm animals mostly focus on their efficiency in pregnancy

TABLE II
The change of plasma OD values of pregnancy-associated glycoproteins in the postpartum period in Konya Merino ewes with single and twin lambing

Postpartum Day	Single lambing	Twin lambing	*r* (Total birth weight and PAG-OD values)
0	0.90 ± 0.14	1.05 ± 0.11	0.545*
3	0.81 ± 0.12	0.99 ± 0.14	0.340
7	0.78 ± 0.09	1.03 ± 0.15	0.569*
10	0.66 ± 0.15	0.93 ± 0.16	0.671**
14	0.42 ± 0.06	0.65 ± 0.12	0.457
21	0.26 ± 0.05	0.42 ± 0.10	0.660**
28	0.08 ± 0.02	0.16 ± 0.04*	0.815***
35	0.05 ± 0.01	0.11 ± 0.02*	0.740***

*:*P*<0.05, **:*P*<0.01, ***:*P*<0.001

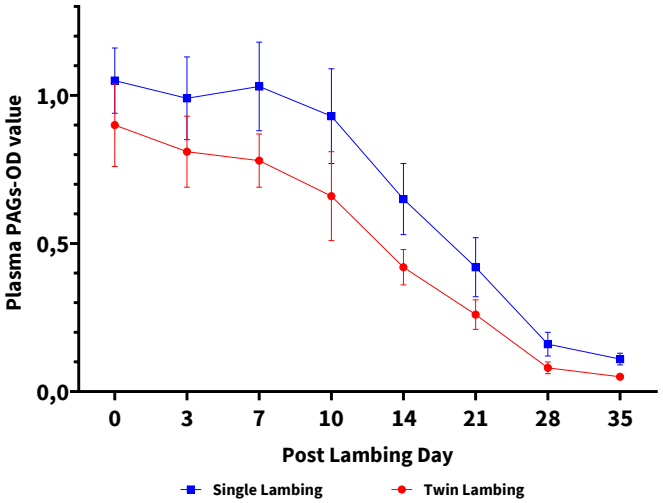

FIGURE 1. The change of plasma OD values of pregnancy-associated glycoproteins in the postpartum period in Konya Merino ewes with single and twin lambing

diagnosis. However, the abundance of these antigens and their expression profiles varying depending on the stage of pregnancy also constitutes relevance in studies regarding their detection and functions in the maternal bloodstream throughout the pregnancy and the postpartum period. In the present study, the plasma profiles of PAGs during the postpartum period were examined in Konya Merino ewes that gave birth to either a single male or female lamb or twin lambs of the same gender. It has previously been shown in several detailed studies on certain breeds that the concentration of pregnancy-associated proteins in maternal plasma increases as the gestational age increases, reaching its highest level at the end of pregnancy in sheep [9, 10, 16, 17]. Ucar *et al*. [17] showed that the concentration of pregnancy-associated proteins in maternal blood increased as the gestational age increased in Konya Merino ewes. In this study, the post-lambing profiles of PAGs, which reached the highest concentration at the end of pregnancy in Konya Merino ewes, were examined and it was found that these antigens were rapidly eliminated from the postpartum maternal blood circulation. The first significant decrease in the mean PAG-OD value during the postpartum period decrease from the plasma mean PAG-OD value on the day of lambing was observed on day 10th postpartum day. The decrease in plasma PAG-OD value in all subsequent weeks was statistically significant when compared to the previous sampling, and it fell below 0.1 on day 35, which was the last day of sampling.

In previous studies, it has been reported that at the end of pregnancy, the concentration of PAG molecules in the blood is higher in twin pregnancies compared to single pregnancies [6, 9]. The present study determined that the plasma PAG-OD value on the day of lambing was higher in ewes with twin lambing, but this difference was not statistically significant. Although Ranilla *et al*. [10] reported similar results in this study, it is generally emphasised in studies conducted on sheep and other species that the PAG concentration in maternal blood circulation is higher in twin pregnancies compared to single pregnancies [6, 9, 18, 19]. In the current study, a significant difference in PAG-OD values between ewes giving birth to single and twin lambs occurred on days 28 and 35 after lambing.

This study determined that the postpartum PAG-OD value in maternal blood of Konya Merino ewes decreased without being associated with lamb gender. Similar results have also been reported in some previous studies conducted on sheep [6, 10].

It has been reported that the residual PAG in the peripheral blood circulation after calving in cows can cause false positive results in pregnancy diagnoses performed after insemination at the end of the voluntary waiting period [20]. It is stated that postpartum PAGs are eliminated from circulation in ewes much faster than in cows, and their half-life is twice as short [12]. It is also noted that the concentration of PAGs in the blood circulation drops to basal levels by the fourth week after lambing [10, 12]. In this study, PAG-OD values dropped below the threshold value of 0.3, which is considered a positive diagnostic value for pregnant cows at this kit analysis, at the 3rd week after lambing in ewes giving birth to a single lamb and at the 4th week after lambing in ewes giving birth to twin lambs. Similarly, Rovani *et al*. [16] determined that the PAG-OD value decreased below 0.3 in the third week of the postpartum period in ewes without specifying the type of lambing in their study using the same kit.

The findings obtained in the current study are generally consistent with the other studies in the literature [10, 12]. However, Roberts *et al*. [9] have stated that the postpartum PAG-OD value dropped below the threshold value of 0.3 in the 8th week postpartum for ewes giving birth to single lambs and in the 9th week postpartum for those giving birth to twin lambs in Polypay x Dorset crossbred ewes. Moreover, previous studies done on goats (*Capra hircus*) and buffaloes (*Bubalis bubalus*) have shown that PAG concentration on the 30th day after birth drops below the threshold value of non-pregnant animals or the value accepted as positive for pregnancy of the same species [21, 22]. Steckeler *et al*. [23] have determined that the optical density values after birth fall below the negative value on the 35th day in whole blood samples, and on the 40th day in plasma or serum samples in ewes. When these results are considered together, it can be seen that the maternal blood PAG concentration is affected slightly by various factors including race, species, sample type, and measurement technique. In contrast to cows, sheep have different reproductive physiology, and they enter a seasonal anestrus and their first natural estrus occurs a long time after giving birth. Therefore, it is emphasised that residual PAGs in the bloodstream would not be a problem for diagnosing a new pregnancy [6, 10]. Even though frequent lambing is applied by inducing postpartum estrus in ewes through hormonal methods, the earliest postpartum inseminations/mating in ewes are usually performed after the weaning of the lambs after a lactation period of approximately 45 days following lambing [6]. As a result, as not applied to induce postpartum estrus in ewes in the current study, the residual PAGs will not be a problem for the diagnosis of a new pregnancy. However, it is also reported that it should not be ignored that there may be false positive results in samples made during or shortly after embryonic death. However, it is also reported that it should not be ignored that there may be false positive results in samples made during or shortly after embryonic death [6, 10].

CONCLUSIONS

The PAG molecules in the peripheral blood circulation after birth in Konya Merino ewes are rapidly eliminated, and the profile of this elimination can be determined with commercial PAG kits. However, new studies can be conducted to use the determination of PAGs molecules and their functions, which are secreted and/or increased in the last period of pregnancy, in the decisions and evaluations to

be taken in the diagnosis, treatment and prophylaxis procedures related to the mother and the offspring (e.g. assessing the quality of colostrum through the quantity of PAGs in it, the survival rate and future growth and development performance of the lambs, among others).

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Ethıcs approval

The study procedure was approved by the Bahri Dağdaş International Agricultural Research Institute Animal Experiments Local Ethics Committee, Konya, Turkey, with protocol number 148 and date 30/11/2022.03).

Conflict of interest

The authors declare that they have no conflict of interest.

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