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RESEARCH ARTICLE

Can Gestational Age be Determined by Placentome Diameter, Placentome Blood Flow Pixel Area and Progesterone Concentration During Pregnancy in Kivircik Ewes?

Sinem Özlem ENGİNLER [1,a (*)] Gamze EVKURAN DAL [1,b]
Ali Can ÇETİN [1,c] Ahmet SABUNCU [1,d] Kerem BAYKAL [2,e]

[1] Istanbul University-Cerrahpasa, Faculty of Veterinary Medicine, Department of Obstetrics and Gynecology, TR-34320 Istanbul - TURKEY
[2] Istanbul University-Cerrahpasa, The Institute of Graduate Education, TR-34320 Istanbul - TURKEY
ORCIDs: [a] 0000-0001-6522-285X; [b] 0000-0002-9996-3290; [c] 0000-0003-4617-8544; [d] 0000-0001-7905-421X; [e] 0000-0003-2200-403X

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Abstract

The information obtained about the gestational age allows ewes to be grouped and fed with appropriate rations for pregnancy. The aims of this study are to evaluate the relationship between placentome diameter (PD), placentome blood flow pixel area (PBFPA) and progesterone (P4) concentrations during pregnancy and to determine which parameter/s is/are effective in determining the gestational age better in sheep whose gestational age is unknown. In the study, 50 singleton pregnant Kivircik ewes were used. The day that the sheep mated with the ram was considered as day 0 of pregnancy. Ultrasonographic examinations and blood sample collections were done on the same day; once a week between 26-40 days, once every two weeks between 41-130 days, and once a week from day 130 of gestation until parturition. The power Doppler images of the biggest 3 placentomes were recorded. ImageJ software program was used for analysis of the area of colored pixels of placentome. The gestational day accurately determined up to 40th, 84th and 96th day of pregnancy according to the PD, PBFPA and P4 concentration, respectively. In conclusion, no relationship (except for PBFPA 40 and PD 40; $P<0.05$) between PD, PBFPA and P4 concentration on same time segments were detected in Kivircik ewes during pregnancy in this study. It was determined that P4 concentration measurements in sheep with unknown breeding time were the most useful parameter for determining the day of gestation, rather than PBFPA and PD, until day 96 of gestation in this study. However, while progesterone allowed for a more accurate determination of the gestational age up to day 96, this method is not yet successful in accurately determining the exact day of gestation beyond day 96 and until the end of pregnancy.

Keywords: *Gestational age, Placentome diameter, Placentome blood flow pixel area, Progesterone, Sheep*

Kıvırcık Koyunlarında Gebelikte Plasentom Çapı, Plasentom Kan Akışı Piksel Alanı ve Progesteron Konsantrasyonu İle Gebelik Yaşı Belirlenebilir mi?

Öz

Gebelik yaşı hakkında elde edilen bilgiler, koyunların gruplanarak gebelik için uygun rasyonlarla beslenmesini sağlar. Bu çalışmanın amacı gebelikte plasentom çapı (PÇ), plasentom kan akımı piksel alanı (PKAPA) ve progesteron (P4) konsantrasyonları arasındaki ilişkiyi değerlendirmek ve gebelik yaşı bilinmeyen koyunlarda gebelik yaşını daha iyi belirlemede hangi parametre/lerin etkili olduğunu tespit etmektir. Çalışmada 50 adet tekiz gebe Kıvırcık koyun kullanıldı. Koyunların koçla çiftleştiği gün gebeliğin 0. günü olarak kabul edildi. Ultrasonografik incelemeler ve kan örneklerinin toplanması 26-40 gün arasında haftada bir, 41-130 gün arasında iki haftada bir, gebeliğin 130. gününden doğuma kadar haftada bir olmak üzere aynı gün yapıldı. En büyük 3 plasentomun power Doppler görüntüleri kaydedildi. Plasentomun renkli piksel alanlarının analizi için ImageJ yazılım programı kullanıldı. PÇ, PKAPA ve P4 konsantrasyonuna göre gebelik günleri sırasıyla 40., 84. ve 96. gebelik günlerine kadar doğru olarak belirlendi. Sonuç olarak, bu çalışmada Kıvırcık koyunlarında gebelik sırasında PÇ, PKAPA ve aynı zaman dilimlerindeki P4 konsantrasyonu arasında (PKAPA 40 ve PD 40 hariç; $P<0.05$) ilişki saptanmadı. Bu çalışmada gebeliğin 96. gününe kadar PKAPA ve plasentom çapından ziyade çiftleşme zamanı bilinmeyen koyunlarda P4 konsantrasyon ölçümlerinin gebelik gününü belirlemede en yararlı parametre olduğu belirlendi. Bununla birlikte, progesteron, 96. güne kadar gebelik yaşının daha doğru bir şekilde belirlenmesine izin verirken, bu yöntem, 96. günden sonra ve gebeliğin sonuna kadar kesin gebelik gününün belirlenmesinde henüz başarılı değildir.

Anahtar sözcükler: *Gebelik yaşı, Plasentom çapı, Plasentom kan akışı piksel alanı, Progesteron, Koyun*

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(*) Corresponding Author

Tel: +90 212 473 7070-17137 Celular Phone: + 90 555 511 7304
E-mail: enginler@iuc.edu.tr (S.Ö. Enginler)

Introduction

Placentomes, approximately 80 per placenta, are the combination of interdigitated fetal cotyledonary and maternal caruncular microvilli; they are also the site of maternal-fetal nutrient exchange in ruminant animals [1-3]. The placentomes can be detected on the surface of the endometrium as particular echogenic areas at days 28 to 30 of gestation by transrectal ultrasonography; as pregnancy further progresses, they maturate into cup-shaped hyperechogenic areas around day 42 in cross-section [4]. Anwar et al.[5] started to examine the placentome scanning on day 26 twice weekly till 60th day of pregnancy transabdominally, using a 3.5 mHZ probe, and observed the maturation of placentomes on days 51 and 55 of pregnancy. Rasheed [6] found that ultrasonographically placentomes can be measured starting from day 35 of pregnancy until day 135, but this measurement is not reliable in determining the gestational age after day 90 of pregnancy. Jones et al.[7] defined perfusion as the volume of blood flowing through a mass of tissue per unit of time. Lemley [8] reported that some tissues and organs can have inadequate amounts of blood flow or the arteries may be too small to maintain their blood flow; therefore the power flow of the Doppler instrument can overcome this difficulty. Power mode is an advanced method of scanning blood flow compared with the traditional color Doppler technique [9], which allows recording blood flow independently of blood flow velocity and direction. This is essential for tissues with low blood flow velocity and numerous blood vessels such as placentomes [10]. Serum progesterone hormone is essential for the maintanance of pregnancy in animals [11,12]. In sheep, serum progesterone has a regular pattern during pregnancy and tends to decline in the prepartum period and at birth [12,13].

For high reproductive efficiency in sheep, basic procedures such as accurate pregnancy diagnosis, determination of gestational age, determination of fetal numbers and removal of non-pregnant females from the herd are required. In particular, the information obtained about the gestation period allows ewes to be grouped and to be fed with appropriate rations according to their nutritional needs for pregnancy; it allows for monitoring females that are close to calving and providing sufficient time to dry off the lactating females [4,14,15].

One of the aims of this study is to evaluate the relationship between PD, PBFPA and P4 concentrations during pregnancy in Kivircik ewes. Another aim of this study is to determine which of these parameter/s is/are effective in determining the gestational age better (until the later day of pregnancy) in sheep whose gestational age is unknown. Thus, with this study, blood flow pixel area in placentomes and placentome diameters during pregnancy of ewes and progesterone concentrations in maternal serum were evaluated.

Material and Methods

Ethical Statement

Ethical approval for the study was obtained from the Istanbul University-Cerrahpaşa Veterinary Faculty Ethics Committee Unit (2019/54, 19.12.2019).

Animals and Management

A total of 170 Kivircik ewes were synchronized with progesterone-based protocol during breeding season and 50 singleton pregnant ewes were selected according to the first pregnancy examination day. Ewes were between 2-6 years old, which were fed semi-intensively. Progesterone-containing intravaginal sponges (30 mg flurorogestone acetate, Chronogest, Intervet, Turkey) were used for 11 days in the breeding season for oestrus synchronization in sheep. Further, 400 IU PMSG (PMSG, Intervet, Turkey) were applied on the day of sponge withdrawal, and fertile rams were added to the sheep herd 48 h later. The day that the sheep mated with the ram was considered as day 0 of pregnancy. Pregnancy diagnosis was made by transrectal ultrasonography between days 17-19 in sheep that were synchronized and were placed with rams, and out of them, 50 singleton pregnant sheep were selected randomly and included to the study.

Study Design

The animals were placed in the standing position, and both sides of the inguinal region in the early period of pregnancy and the more cranial side of the ventral abdomen in later periods were shaved; then the probe was positioned to display the maximum size of the placentomes ultrasonographically (Esaote, MyLab One, Italy). In the current study, the biggest three placentomes were measured, and mean diameter of these placentomes were calculated in centimeters (cm). Ultrasonographic examinations were conducted with a 5 mHz microconvex probe transabdominally once a week between days 26 to 40, once every two weeks between days 41 to 130, and once a week from day 130 of gestation until parturition. In order to obtain the maximum number of colored pixels captured in power Doppler examination in the placentomes, the probe was adjusted in place by light manipulation before freezing *(Fig. 1)*. Using the power flow function of the Doppler ultrasound, 3 placentomes were imaged transabdominally for blood perfusion. Ultrasound images were uploaded from the Esaote, MyLab One ultrasound device and a software program was used for analysis of the area of colored pixels of placentome tissue in sheep in gravid horn. Ultrasound images firstly were set scaled from pixels to mm, and then the placentome was selected via the freehand tool then the images were edited to distinguish the blood perfusion area, the surroundings of the placentome were all being cleared with "clear outside" tool. The colored perfusion areas were saturated with threshold function of ImageJ program (ImageJ, version

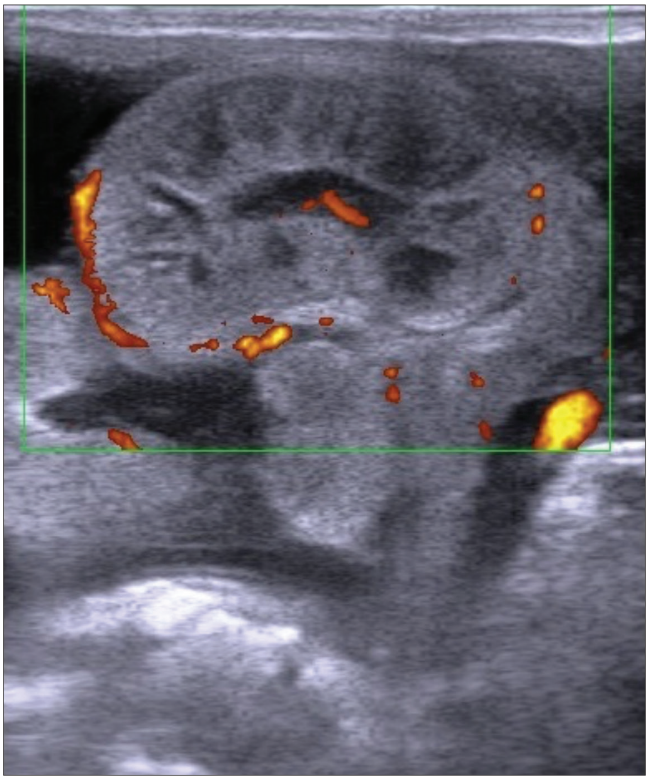

Fig 1. The ultrasound probe was adjusted in place by light manipulation before freezing, and maximum number of colored pixels was captured in a power Doppler examination in the placentomes

1.50e, US National Institutes of Health, USA). These areas were measured using the "Analyse" function of ImageJ for each placentome. From these data, the mean pixel areas of the biggest three placentomes were recorded for each animal in mm^2.

On the ultrasonographic examination days, 10 mL blood samples were collected from the jugular vein into anti-coagulant tubes. Blood samples were centrifuged at 1000 x g for 10 min for serum retrieval and the obtained sera were kept at -20ºC until progesterone analysis. The progesterone concentration in serum samples were determined by the quantitative sandwich enzyme immunoassay technique using commercial kits (Sheep progesterone ELISA, Bioassay Technology Laboratory, Shanghai, China) according to the manufacturer's instructions with a sensitivity of 0.027 ng/mL. The intra- and inter-assay coefficients of variation for the kit were <8 and <10%.

Statistical Analysis

The relationship between placentome diameter, placentome blood flow pixel area and progesterone concentration were analyzed using the Pearson correlation analysis method.The Shapiro-Wilk test was used to check the normality of data. Statistical analyses of the parameters were measured with ANOVA for, repeated measures. The SPSS 13.0 package program was used for analysis. For all statistical analyses performed, P<0.05 was accepted as significant.

Results

Although there was statistical significance (P<0.05) between some features in general, a relationship that allow to make a meaningful interpretation could not be determined. Besides, according to the same time segments no relationship among PD, PBFPA and P4 concentration were detected in Kivircik ewes during pregnancy in this study, except for PBFPA 40 and PD 40 (P<0.05) *(Table 1)*. In this study, it was observed that placentome diameters increased until day 84 of gestation and reached its maximum value (3.69 cm) on day 84. Then, it decreased to 1.57 cm by day 145 of gestation. Although the gestational day can be accurately determined up to day 40 of pregnancy according to the PD, the exact day of gestation beyond day 40 cannot be determined. Placentome blood flow pixel area increased until day 124 of gestation and reached its maximum value (39.46 mm^2). From that point, it started to decrease and reached to its final value 21.92 mm^2 by the end of pregnancy. According to the PBFPA, it is evident that until day 84 of pregnancy, gestational age can be accurately determined, but after that point, the exact day of pregnancy cannot be clearly determined till the end of gestation. Based on the PBFPA measurement, gestational age can only be established as lying between days 84 to 145.

In this study, P4 concentration in maternal serum was found to increase until day 124 of gestation with its maximum concentration at 13.85 ng/mL, and from that point on, the P4 concentration decreased (11.50 ng/mL) at the end of gestation. It was observed that, based on the P4 concentration, the pregnancy can be accurately determined until day 96 of pregnancy. By obtaining the P4 concentration in pregnancies after 96 days, the pregnancy can be foreseen only between 96-145 days. According to the statistical analysis, the effects of the features depending on the time of pregnancy were found to be different (P<0.001) *(Table 2)*.

Discussion

Pregnancy might be recognized as early as 17-19 days post breeding using B-Mode ultrasonography in sheep [16,17]. Jyothi et al.[18] observed placentomes first on day 22 of gestation by transabdominal ultrasonography as an echogenic dense line in Nellore brown ewes. Due to the visualization of placentomes by transabdominal ultrasonography, we performed ultrasonographic examinations on day 26 and further until the end of gestation to predict the most effective parameter --PD, PBFPA and/or P4 concentration-- to detect the exact gestational age in the ewes in this study. According to the same time segments, only one positive relationship between PBFPA 40 and PD 40 (P<0.05) was found. However, it would not be correct to reveal a meaningful relationship between the study parameters by determinig at this result alone. It was reported that the mean diameters of placentomes

Table 1. *Correlation analysis of placentome diameter, placentome blood flow pixel area and progesterone concentration*

Parameter	PBFPA 26	PBFPA 33	PBFPA 40	PBFPA 54	PBFPA 68	PBFPA 84	PBFPA 96	PBFPA 110	PBFPA 124	PBFPA 131	PBFPA 138	PBFPA 145	P 26	P 33	P 40	P 54	P 68	P 84	P 96	P 110	P 124	P 131	P 138	P 145	PD 26	PD 33	PD 40	PD 54	PD 68	PD 84	PD 96	PD 110	PD 124	PD 131	PD 138	PD 145
PBFPA26	1.0	0.7	0.0	-0.2	0.1	0.0	0.0	0.0	0.1	-0.1	0.0	-0.1	-0.1	-0.1	-0.2	-0.2	0.0	-0.1	0.2	0.0	0.3	0.1	0.1	-0.2	0.1	0.2	**0.3**	**0.3**	0.1	0.1	-0.1	0.1	0.1	0.1	0.1	0.0
PBFPA33		1.0	0.3	0.0	0.2	0.1	0.1	0.1	-0.2	0.1	0.1	0.1	-0.2	0.0	0.0	-0.1	-0.1	-0.1	0.1	**0.4**	**0.5**	**0.3**	0.2	-0.3	-0.1	0.2	**0.3**	0.2	-0.1	0.0	-0.1	0.0	0.1	0.1	0.1	0.0
PBFPA40			1.0	**0.3**	**0.4**	**0.3**	**0.4**	**0.3**	-0.2	0.2	-0.1	0.0	-0.1	0.0	0.0	-0.2	-0.1	0.1	0.2	0.1	0.3	0.2	0.2	-0.3	-0.1	**0.4**	**0.3**	0.2	0.0	0.2	0.1	0.0	0.0	0.1	0.1	0.0
PBFPA54				1.0	0.1	0.1	0.1	-0.1	0.0	0.0	0.1	0.1	-0.1	0.1	0.0	-0.1	-0.1	0.3	0.1	0.2	0.1	0.1	0.1	0.0	0.0	0.2	-0.1	0.0	-0.1	-0.1	-0.1	-0.1	0.0	0.0	0.0	-0.1
PBFPA68					1.0	**0.4**	0.1	0.0	**-0.4**	0.1	-0.1	0.0	0.0	0.1	0.0	0.1	-0.1	0.0	0.2	0.1	0.3	0.2	0.2	-0.1	-0.1	**0.3**	**0.4**	**0.4**	0.1	0.3	0.1	0.0	-0.2	-0.2	-0.2	-0.2
PBFPA84						1.0	0.2	0.1	**-0.3**	0.0	0.0	-0.1	0.0	-0.1	-0.2	0.1	-0.2	0.1	0.1	0.2	**0.3**	0.2	0.2	-0.2	-0.1	0.2	0.2	0.0	0.0	0.2	0.0	-0.1	-0.2	-0.2	-0.1	-0.1
PBFPA96							1.0	0.2	-0.1	0.2	0.0	-0.2	0.1	0.1	0.2	0.1	-0.1	0.2	0.1	0.1	0.2	**0.3**	**0.3**	0.0	0.0	0.1	0.2	0.2	0.1	0.2	0.0	0.2	0.0	0.0	-0.1	-0.2
PBFPA110								1.0	0.0	0.2	0.1	-0.2	0.2	-0.1	0.0	0.0	0.1	0.0	-0.2	-0.1	0.1	0.0	0.1	-0.1	0.2	0.0	0.2	-0.1	-0.1	-0.1	-0.2	-0.1	-0.3	-0.3	**-0.3**	**-0.3**
PBFPA124									1.0	-0.2	-0.1	0.0	0.0	-0.1	0.0	-0.1	0.2	0.1	0.1	0.0	0.0	0.1	0.1	0.1	0.0	-0.1	-0.3	-0.1	0.1	-0.1	-0.1	-0.2	-0.1	-0.1	-0.1	0.0
PBFPA131										1.0	0.0	0.0	0.0	0.1	0.1	0.1	0.0	-0.1	0.0	0.1	0.2	0.3	0.3	0.1	-0.1	-0.1	-0.2	0.1	0.1	0.1	0.1	0.3	0.1	0.1	0.1	0.0
PBFPA138											1.0	**0.5**	-0.1	-0.1	0.0	-0.1	-0.2	-0.2	-0.2	0.0	0.0	0.0	0.0	0.2	0.1	-0.1	**-0.4**	**-0.3**	-0.1	-0.1	0.0	-0.1	0.0	0.0	-0.1	-0.2
PBFPA145												1.0	**-0.3**	0.0	-0.1	0.0	0.1	-0.1	-0.1	0.0	0.0	-0.1	-0.1	-0.1	-0.2	-0.1	-0.2	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	-0.1
P26													1.0	**0.5**	**0.4**	0.2	0.0	0.0	0.0	-0.2	-0.2	-0.1	0.0	0.1	0.2	0.0	0.0	0.0	0.0	-0.1	-0.1	-0.1	0.0	0.0	0.0	0.0
P33														1.0	**0.8**	**0.3**	0.1	0.0	0.0	0.0	0.0	0.0	-0.1	0.1	0.1	0.0	0.1	0.3	0.0	0.1	-0.1	-0.1	0.0	0.0	0.0	-0.1
P40															1.0	**0.3**	0.1	-0.1	-0.1	0.0	0.0	0.0	0.0	**0.3**	0.1	0.0	0.1	0.2	0.1	0.1	-0.1	0.0	0.1	0.0	-0.1	-0.1
P54																1.0	0.2	0.2	0.1	0.0	0.1	0.0	0.1	0.2	0.0	0.0	0.1	**0.4**	0.1	0.2	0.2	0.1	0.1	0.1	0.1	0.0
P68																	1.0	0.0	0.0	-0.1	0.0	0.0	0.1	0.2	-0.1	-0.3	0.0	0.1	0.2	0.1	-0.1	0.0	0.0	0.0	0.0	0.0
P84																		1.0	**0.5**	0.2	0.1	0.1	0.1	-0.1	-0.2	-0.2	0.0	0.1	-0.3	-0.2	-0.2	-0.3	-0.2	-0.2	-0.2	-0.2
P96																			1.0	**0.5**	**0.4**	**0.5**	**0.4**	0.0	**-0.4**	-0.1	-0.1	0.1	0.0	0.1	0.1	-0.1	0.0	0.0	0.0	-0.1
P110																				1.0	**0.7**	**0.7**	**0.6**	0.0	**-0.4**	-0.2	-0.1	-0.2	0.0	0.1	0.0	-0.1	0.0	-0.1	-0.1	-0.2
P124																					1.0	**0.8**	**0.8**	-0.1	-0.2	0.1	0.1	0.2	0.2	**0.4**	0.1	0.1	0.0	0.1	0.0	-0.1
P131																						1.0	**0.9**	0.1	-0.2	0.0	0.0	0.0	0.3	**0.3**	0.1	0.0	0.0	0.0	0.0	-0.1
P138																							1.0	0.1	-0.2	0.0	0.0	0.1	**0.3**	**0.4**	0.1	0.1	0.1	0.1	0.0	0.0
P145																								1.0	0.1	-0.2	**-0.3**	-0.1	0.0	0.0	0.0	0.1	-0.1	-0.1	-0.1	-0.1
PD26																									1.0	**0.6**	**0.3**	0.1	0.2	0.1	0.1	0.2	0.1	0.2	0.2	0.2
PD33																										1.0	**0.7**	**0.5**	0.2	**0.4**	**0.4**	**0.3**	0.2	0.2	0.2	0.3
PD40																											1.0	**0.5**	0.2	**0.3**	0.3	0.3	0.0	0.1	0.0	0.1
PD54																												1.0	**0.3**	**0.5**	**0.3**	**0.3**	**0.3**	0.3	0.3	0.2
PD68																													1.0	**0.9**	**0.7**	**0.6**	**0.5**	**0.5**	**0.5**	**0.5**
PD84																														1.0	**0.7**	**0.6**	**0.4**	**0.5**	**0.5**	**0.4**
PD96																															1.0	**0.7**	**0.6**	**0.6**	**0.6**	**0.5**
PAD110																																1.0	**0.7**	**0.7**	**0.6**	**0.6**
PD124																																	1.0	**1.0**	**0.9**	**0.8**
PD131																																		1.0	**1.0**	**0.8**
PD138																																			1.0	**0.9**
PD145																																				1.0

Those written in bold are statistically significant (P<0.05); PBFPA: Placentome blood flow pixel area, P: Progesterone concentration, PD: Placentome diameter

Table 2. *The results of mean placentome diameter, placentome blood flow pixel area and progesterone concentration throughout pregnancy in Kivircik ewes*

Gestational Days	Placentome Diameter Mean ± SE (cm)	Placentome Blood Flow Pixel Area Mean ± SE (mm^2)	Progesterone Concentration Mean ± SE (ng/mL)
26	0.72±0.03^{a}	1.10±0.10^{a}	2.41±0.08^{a}
33	0.95±0.02^{b}	2.09±0.08^{b}	2.89±0.08^{b}
40	1.23±0.03^{c}	4.60±0.10^{c}	3.24±0.07^{c}
54	2.33±0.06^{g}	8.03±0.07^{d}	6.58±0.10^{d}
68	3.33±0.06^{i}	11.25±0.12^{e}	8.52±0.10^{e}
84	3.69±0.07^{j}	17.20±0.15^{f}	11.33±0.14^{f}
96	2.77±0.05^{h}	25.73±0.26^{h}	12.39±0.09^{g}
110	2.25±0.04^{g}	32.89±0.37^{j}	13.46±0.06^{i}
124	1.79±0.02^{f}	39.46±0.21^{k}	13.85±0.07^{j}
131	1.74±0.02^{ef}	30.66±0.19^{i}	13.50±0.06^{i}
138	1.66±0.02^{de}	21.61±0.18^{g}	13.06±0.06^{h}
145	1.57±0.02^{d}	21.92±0.21^{g}	11.50±0.05^{f}
P	P<0.001	P<0.001	P<0.001

$^{a-k}$ *The significant differences between time in the columns is shown in different letters*

as approximately 3.0 cm and remained unchanged from the first scanning (day 60 of pregnancy) until lambing on Santa Ines ewes with 9 females carried twins and 4 ewes had single pregnancies [19]. Mean PDs in the similar examining days were detected lower in ewes carrying single offspring in this study. In another study, Dwyer et al.[20] reported that placental weight, placental cotyledons and mean placental weight were lower in singleton pregnant sheep than in twins. Placentomes began to appear as buds on the endometrium surface, giving an echogenic appearance on day 28 of pregnancy in Merinos ewes [15]. Doize et al.[4] detected the placentomes on days 28-30 ultrasonographically in their study in mixed-breed ewes. In this study, the ultrasonographic examinations were started on day 26 after controlled mating, and the placentomes were detected as buds giving an echogenic appearance on this day - results reported by Aydın et al.[15] and Doize et al.[4] were very close to those results also. Alexander [21] observed that placentome sizes can vary in relation to their location in the uterine horn, where the smallest placentomes were found at the tips of the horns and the largest near the junction of both horns. In this study, placentome diameter increased and until day 84 of gestation, after which their size started to decrease; the maximal size of the placentomes was recorded on day 84 of gestation. Doize et al.[4] reported the maximum size of the placentomes detected by transrectal ultrasonography method on day 74 of gestation. The variation of placental development in relation to season has also been reported previously [22], the number of developed placentomes and their total weight was much greater in females bred during the normal breeding season than those bred during the anestrus season [4]. In the present study, examinations were performed on females bred during the normal breeding season in line with a previous study [4]. Waziri et al.[23] reported a high correlation between PD and gestatinal age in sheep. However, the mean diameter of placentomes was not statistically different during the last 7 weeks before parturition. The authors concluded that PD measurement can be used as an indicator of gestational age only at midgestation. In the present study, the gestational day can be accurately determined up to day 40 of pregnancy according to the PD.

Doppler ultrasonography is as extremely useful tool for small ruminant reproduction [24]. Power Doppler sonography is a new scanning method that displays the strength of the Doppler signal in color rather than the speed and direction of blood flow. Therefore, it is approximately three times more sensitive than a conventional color Doppler for detecting blood flow; therefore especially useful for those with small blood vessels and low-velocity flow [25]. In a study [26], pregnancy can be diagnosed accurately by semiquantitative assessment of corpus luteum vascularization as early as 17 days post breeding in ewes. Besides, placentome blood flow pixel area can be detected by power Doppler ultrasonography as this method facilitates the evaluation of capillary blood flow. Still images or recordings obtained during power/color Doppler examinations can be stored for later for analysis with various image analytical programs. The vascularized (colored) area of an ultrasonogram can be taken as a proportion or percentage of the total cross-sectional area of the organ or tissue, and the pixels corresponding to blood flow velocities can be measured [25,27,28]. In this study, PBFPA was measured using a software program to eliminate the errors that may arise from subjective evaluations. The vascularization of placentome tissue is an important feature for the gestational process, and the increase in blood flow in these structures throughout pregnancy is related to the nutritional requirements of the foetus and the proximity of parturition [29]. Lemley et al.[30] examined the changes in placentome blood perfusion that occurred due to vasoactive medications and determined acute fluctuations in placentome blood perfusion in singleton pregnant ewes. Da Silva et al.[31] evaluated fetomaternal structures throughout pregnancy by ultrasonography in ewes. The values achieved from gray-scale analysis of the placentomes decreased gradually. As gestation progressed, the echogenity of placentomes changed from hyperechoic to hypoechoic structures, especially from the 18th week of pregnancy. However, the echotexture of the tissue remained homogeneous. Although Doppler ultrasonography was not performed, the reduction of placental echogenicity was suggested to be associated with an increase in the blood volume, which is in line with the highest pixel area calculated on day 124 in our study.

Progesterone with other hormones directly related to pregnancy, and P4 is necessary for the continuation of pregnancy in domestic animals, including sheep. Hamon and Heap [32] reported an increase during the early gestational period and a reduction in the P4 concentration between days 30 to 50; afterwards the P4 concentration was found to increase again. Kalkan et al.[33] reported a mean P4 concentration of 2.02 ng/mL begininig from 30 day of gestation in blood samples collected every 20 days till parturition in sheep with single offspring, besides they indicated that when the number of fetuses increases, P4 level increases in maternal serum and also P4 measurement can be used to predict the fetal number after the second half of pregnancy. Progesterone concentration is approximately 10.02 ng/mL during the same gestational period in current study. Yotov [34] reported P4 concentrations of 15.0 ng/mL and 19 ng/mL and 17.3 ng/mL and 23.3 ng/mL in Trakia Merino and Pleven Blackhead sheep on days 40 and 60, respectively, during pregnancy. In this study, the P4 concentration was found to be 3.24 ng/mL and 8.52 ng/mL on days 40 and 68 of gestation, respectively, in Kivircik ewes. The P4 concentration was observed to increase until day 124 of gestation, and it was measured as 13.85 ng/mL at that point, after which it decreased until parturition in this study. Manalu and Sumaryadi [35] reported the mean P4 concentration as 16.9 ng/mL between weeks 8-20 of pregnancy in sheep with a single fetus. Dramatic increase in maternal serum P4 concentration during the fetal phase (8-20 week) of pregnancy is due to the role of placental P4 [36,37]. The P4 concentration measured by RIA method in their study was higher during the same gestational period. Stabenfeldt et al.[38] reported a decline in P4 concentration beginning around days 130-135 (week 19) and continued to the day of parturition; this suggests that endocrine preparation for delivery begins about 2 weeks prior to parturition. The P4 concentration started to decrease on day 131 and continued to the day of parturition in our current study, which is in line with their study.

In conclusion, according to the same time segments, only one positive relationship between PBFPA 40 and PD 40 was found. It was associated with the increased blood supply due to the increase in placentome diameter. Although, statistically significant differences by time were found in terms of PD, PBFPA and P4 concentration, according to the results of this study, it was determined that P4 concentration measurements in sheep with unknown breeding time were more useful parameters in determining the day of gestation than PBFPA or PD. However, while progesterone allowed for a more accurate determination of the gestational age up to day 96, this method is not yet successful in accurately determining the exact day of gestation beyond day 96 and until the end of pregnancy.

Availability of Data and Materials

The datasets during and/or analyzed during the current study available from the corresponding author on reasonable request.

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Conflict of Interest

The authors declare that they have no conflict of interests.

Author Contributions

S.Ö. Enginler: Project administration, conceptualization, methodology, software, visualization, investigation, writing - original draft, writing- review & editing. G. Evkuran Dal: Methodology, visualization, data curation, software. A.C. Çetin: Conceptualization, investigation, data curation, software. A. Sabuncu and K. Baykal: Methodology, investigation, visualization.

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