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Evaluation of changes in Doppler ultrasonography indices and levels of maternal serum angiogenic factors throughout pregnancy in ewes

Özge Turna Yilmaz*, Mehmet Can Gündüz, Gamze Evkuran Dal, Melih Uçmak, Zeynep Günay Uçmak, Esra Karaçam, Güven Kaşikçi, Mehmet Ragip Kiliçarslan

Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Istanbul University, Istanbul, Turkey

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ABSTRACT

We evaluated the changes in the pulsatility index (PI) and resistance index (RI) of fetal and maternal vessels and maternal serum angiogenic factors throughout pregnancy in 20 Kivircik ewes. They were examined for pregnancy detection on Day 30 after mating. The PI and RI during the pregnancies were followed using Doppler ultrasonography (USG) of the uterine artery, umbilical artery, fetal aorta, and fetal vena cava. Doppler USG was performed every 15 days beginning from the 40th day after mating. Blood samples were collected every 15 days starting from the day of mating. Nitric oxide (NO), vascular endothelial growth factor, basic fibroblast growth factor, and angiopoietin-1 and -2 levels were measured in maternal serum. A gradual decline was observed in the PI and RI of the umbilical artery beginning from Day 75 of pregnancy. Similarly, the RI of the uterine artery decreased on Day 135. Embryonic resorption was detected in two ewes with an increased PI and RI in the uterine artery. A significant and gradual decrease in the maternal serum vascular endothelial growth factor level was observed throughout pregnancy. The maternal serum NO level increased beginning from Day 135 of pregnancy. The results of this study illustrate the progressive changes in the Doppler USG findings of fetomaternal vessels and maternal serum angiogenic factors that occur throughout ewe pregnancy. The umbilical and uterine artery Doppler USG findings and maternal serum NO concentration may be important parameters for evaluation of the course of pregnancy in ewes. The results of this study should be compared with those of further studies that include compromised pregnancies and nonpregnant ewes.

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1. Introduction

Advances in reproductive technology have led to techniques such as embryo transfer and cloning in animal breeding. However, some studies have revealed that abortions and disorders in placental and/or fetal development are common during both early and advanced stages of these kinds of pregnancies [1,2], requiring follow-up of fetal and placental development. However, the knowledge of fetal and placental development in normal and compromised pregnancies in ewes is limited. Few published studies have evaluated the Doppler ultrasonography (USG) findings in pregnant small ruminants. Serin et al. [3] reported that the pulsatility index (PI) and resistance index (RI) of the umbilical artery decreased in the middle of the second trimester in a pregnant goat. In another study [4], the applicability of noninvasive Doppler USG in pregnant small ruminants was investigated. The authors stated that the PI and RI of the uterine artery decreased until parturition.

* Corresponding author. Tel.: +902124737070/17315; fax: +90(212) 5190834.
E-mail address: turnao@istanbul.edu.tr (Ö.T. Yilmaz).

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The uterine and placental blood flow increases during pregnancy to supply the metabolic requirements of the growing fetus [5]. Therefore, tracking blood flow during pregnancy would enable us to obtain information about fetal development. Doppler USG is an imaging method that provides valuable information about the physiological and pathological differences in circulation between the mother and fetus [6]. Abnormal vascular findings in fetal and/or maternal compartments may be indicators of intrauterine growth restriction, fetal distress, or early pregnancy failure [7,8].

New vessels develop in the uterus to supply the increasing metabolic requirements of the fetus during pregnancy [9]. The formation of new blood vessels is called angiogenesis [10]. Angiogenesis is initiated by the actions of vascular endothelial growth factor (VEGF), placental growth factor, basic fibroblast growth factor (bFGF), and tumor necrosis factor-alpha [11]. However, VEGF, bFGF, and angiopoietins (ANGPs) are the most potent positive regulators of this process [12]. Nitric oxide (NO) is another regulator that acts on placental and fetal blood flow. It plays a central role in the endometrium during embryo implantation and in the uterus and cervix at the onset of labor [13,14].

The umbilical and uterine artery blood flow, oxygen-bearing capacity of the fetal and maternal blood, placental surface area, placental permeability, and quality of oxygen consumed by the placenta are major factors that affect the transport of oxygen to the fetus [15]. Placental oxygen diffusion is minimal during early pregnancy. However, gas transport begins with the progression of pregnancy and maturation of the placenta's vascularity [16]. This is why placental angiogenesis is so important for fetal development.

The aim of this study was to evaluate the changes in the PI and RI of the fetal arteries and uterine artery and various angiogenic factors in the maternal serum throughout pregnancy in ewes.

2. Materials and methods

2.1. Animals and study design

Twenty-five Kivircik multiparous pregnant ewes were used in this study. Estrus was synchronized, and each ewe was allowed to mate with a ram (one ram per five ewes) during estrus. Ethical approval for the study was obtained from the Istanbul University Animal Research and Ethical Committee (2012/03, 20.01.2012). The ewes were examined for pregnancy diagnosis 30 days after mating using transrectal B-mode USG (Esaote Pie Medical MyLab Five Vet, 5-MHz linear transducer; Esaote Pie Medical, Genoa, Italy). Transabdominal Doppler USG (Esaote Pie Medical Five Vet, 6.6-MHz microconvex transducer; Esaote Pie Medical) was performed every 15 days from Day 45 to 135 of pregnancy. The examinations were performed with the ewe in dorsal recumbency until 90 days of pregnancy, after which time the ewes were placed in lateral recumbency to prevent maternal aorta compression. Hair was clipped from the inguinal and abdominal regions before USG examination, and ultrasound gel was used to avoid intense sound reflections caused by air pockets at the border between the transducer and skin. The insonation angle was greater than 20° for all examinations. At least three consecutive systolic peaks with similar velocity and amplitude were used for analysis. The profile of the second pulsation was subjected to analysis of the PI and RI of the fetal aorta, fetal vena cava, umbilical artery, and uterine artery. The indices were measured individually in twin fetuses. The fetal vena cava near the right atrium was visualized with its characteristic waveform. The fetal aorta was scanned in the aortic arch, and the free-floating umbilical cord with two veins and two arteries was visualized around the fetus. The uterine artery was visualized near the uterine wall of the gravid uterus as described by Elmetwally and Meinecke-Tillmann [4]. Maternal blood samples were collected from the jugular vein of the ewes before mating and during USG examinations to detect maternal serum angiogenic factors. Serum samples were stored at −20 °C until analysis. Concentrations of VEGF, bFGF, ANGP-1, and ANGP-2 were assayed with enzyme-linked immunosorbent assay kits (USCN Life Science Inc., Wuhan, China), and NO levels were measured using colorimetric assay kits (Cayman Chemical Company, Ann Arbor, MI, USA), following the manufacturer's instructions. According to the manufacturer, mean minimal detectable doses are 2.1 pg/mL for VEGF, 4.23 pg/mL for fibroblast growth factor (FGF), 0.06 ng/mL for ANGP-1, 4.1 pg/mL for ANGP-2, and 2.5 μM for NO. Nitrate in the samples was reduced to nitrite by nitrate reductase enzyme, and the total nitrite was determined by Griess method [17], which converts nitrate into a deep purple azo compound. The total amount of NO was calculated with the sum of nitrite and nitrate.

2.2. Statistical analysis

Repeated-measures ANOVA was used to determine the differences in each parameter throughout pregnancy. Student's *t* test was used to test for any significant differences between single and twin pregnancies throughout pregnancy. An interaction analysis was performed between the two main factors: multiple pregnancy and time. The data distribution was analyzed with the Shapiro–Wilk test. A parametric test was used for data with a normal distribution, whereas a nonparametric test was used for data with a non-normal distribution. The Mann–Whitney U test was used as a nonparametric test to analyze the differences between single and twin pregnancies. The effect of sampling time was analyzed using the Friedman and Wilcoxon tests. Data were analyzed using SPSS version 10 (SPSS Inc., Chicago, IL, USA). For all statistical analyses performed, P value less than 0.05 was accepted as significant. Data are expressed as the mean ± standard deviation.

3. Results

Twenty single and five multiple pregnancies were detected on the 30th day of pregnancy by transrectal USG. A tentative diagnosis of embryonic resorption was made on Day 45 of pregnancy in two ewes. These ewes were excluded from the study. Fifteen single and 10 twin healthy, normal-birthweight lambs were born in the study. No other cases of intrauterine growth retardation (IUGR) or abortion were recorded during the study.

3.1. Doppler USG results

3.1.1. Fetal aorta PI and RI

Only systolic waveforms could be detected by USG until Day 75 of pregnancy. The effect of the interaction between the number of fetuses and day of pregnancy on the fetal aorta PI and RI was not statistically significant ($P > 0.05$). The time-dependent changes in the PI ($P < 0.05$; Fig. 1A) and RI ($P < 0.001$; Fig. 1B) of the fetal aorta were significant but were similar between single and twin pregnancies. There was a significant decrease in the RI of the fetal aorta from 60 days of pregnancy.

3.1.2. Fetal vena cava PI and RI

The effect of the interaction between the number of fetuses and day of pregnancy on the fetal vena cava PI and RI values was not statistically significant ($P > 0.05$). The time-dependent changes in the PI ($P < 0.001$; Fig. 2A) and RI ($P < 0.001$; Fig. 2B) of the fetal vena cava were significant but were similar between single and twin pregnancies. A significant reduction on fetal vena cava PI and RI was first observed at Day 90 of pregnancy.

3.1.3. Umbilical artery

Measurement of the end-diastolic velocity of the umbilical artery was first possible on Day 90 of pregnancy. No pathology of umbilical vein pulsation was observed in the ewes. The effect of the interaction between the number of fetuses and day of pregnancy on the umbilical artery PI and RI was not statistically significant ($P > 0.05$). There were significant time-dependent changes in the PI (Fig. 3A) and RI (Fig. 3B; both $P < 0.001$) of the umbilical artery, but they were similar between single and twin pregnancies. A significant decrease in both the PI and RI was seen toward the end of pregnancy.

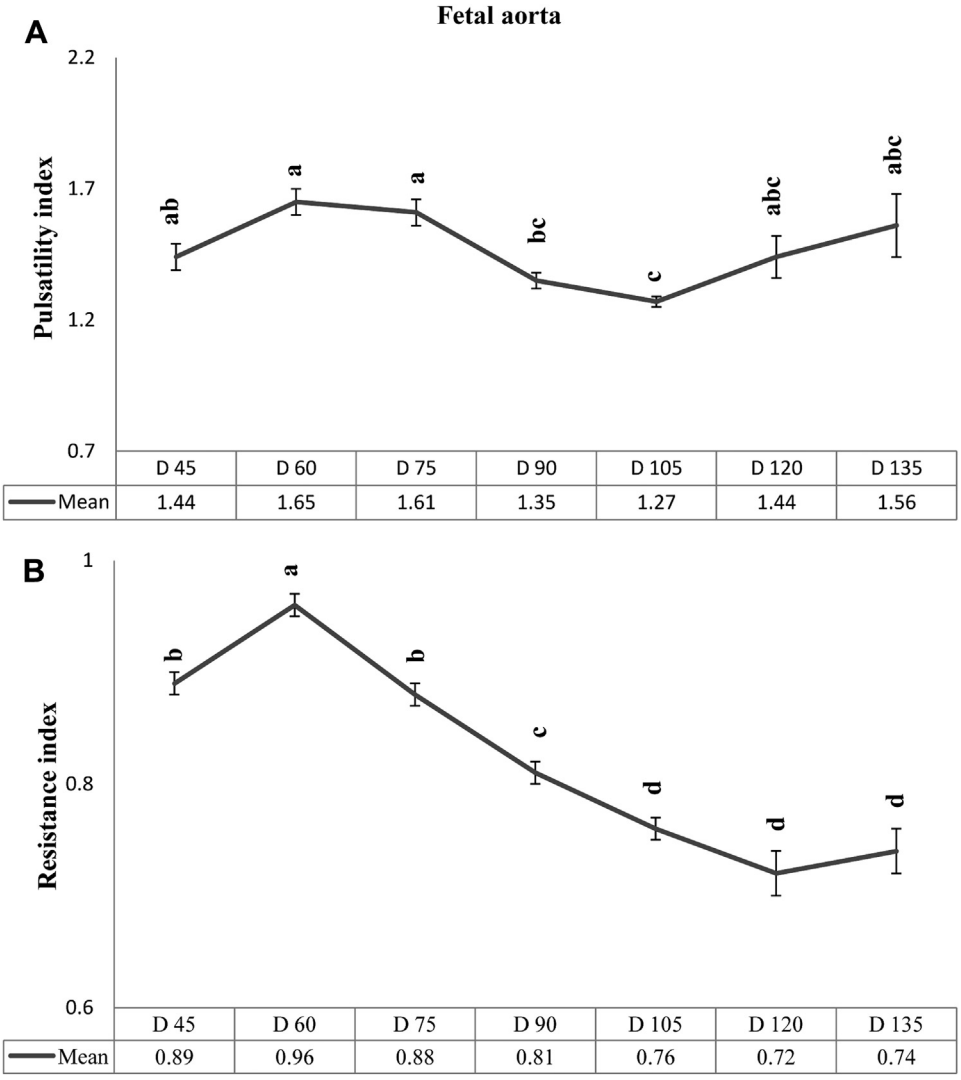


Fig. 1. Time-dependent changes in pulsatility index (A) and resistance index (B) values of the fetal aorta throughout pregnancy. Different letters above the graph points indicate significant differences.

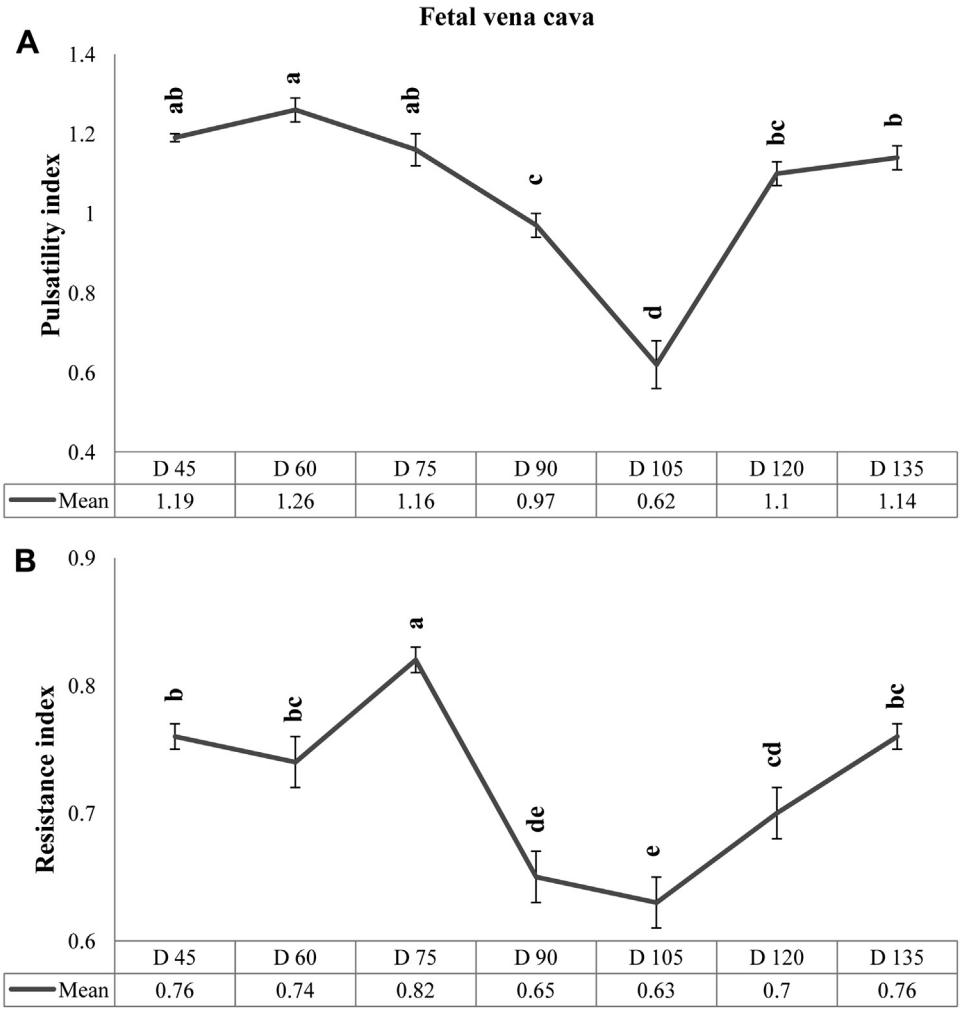


Fig. 2. Time-dependent changes in pulsatility index (A) and resistance index (B) of the fetal vena cava throughout pregnancy. Different letters above the graph points indicate significant differences.

3.1.4. Uterine arteries

The PI and RI of two ewes that were diagnosed with embryonic resorption on the 45th day of pregnancy were 1.76 and 2.06, respectively, in one ewe and 0.79 and 0.81, respectively, in the other. These exceeded the overall mean PI and RI for the remaining ewes. A pathological diastolic notch was observed in the uterine artery waveform of one of these ewes (Fig. 4).

The effect of the interaction between the number of fetuses and day of pregnancy on the uterine artery PI and RI was not statistically significant ($P > 0.05$). There were significant time-dependent changes in the PI (Fig. 5A) and RI (Fig. 5B; both $P < 0.05$), but they were similar between single and twin pregnancies.

3.2. Maternal serum angiogenic factors

According to the results of the Shapiro–Wilk test, the maternal serum NO data were normally distributed, whereas the maternal serum VEGF, FGF, ANGP-1, and ANGP-2 data were non-normally distributed. The effect of the interaction between the number of fetuses and day of pregnancy on the maternal serum NO concentration was not statistically significant ($P > 0.05$). The effect of pregnancy type on the maternal serum VEGF, FGF, ANGP-1, and ANGP-2 concentrations was not significant according to the results of the Mann–Whitney U test.

The time-dependent changes in each parameter are shown in Figure 6. A significant increase in the serum NO concentration was seen beginning from Day 135 of pregnancy. ANGP-1 reached its lowest concentration on Day 105 of pregnancy.

4. Discussion

Thompson et al. [18] suggested that the flow parameters in the thoracic aorta of lamb fetuses are similar to those of human fetuses; therefore, studies carried out with these two species complement each other. There are a limited number of studies on fetal Doppler USG in ewes, especially

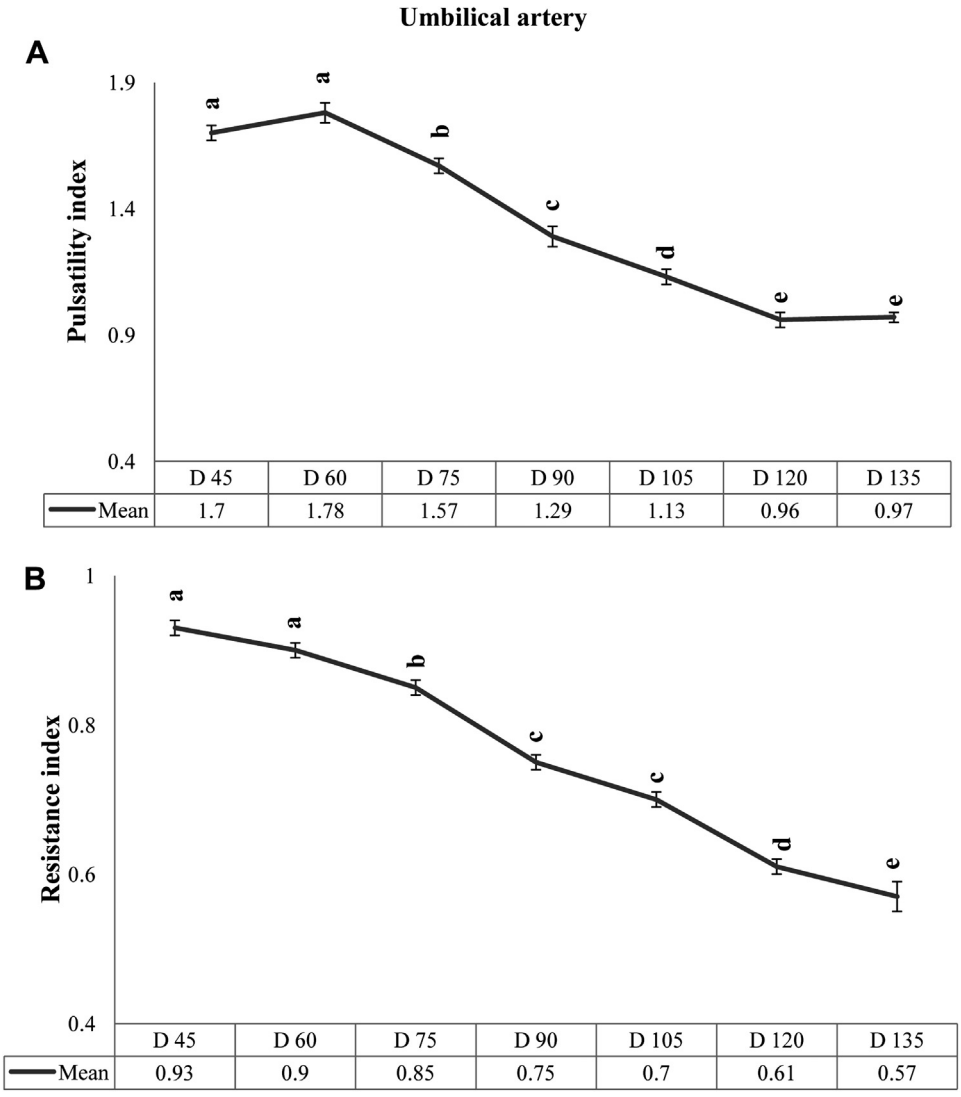


Fig. 3. Time-dependent changes in the pulsatility index (A) and resistance index (B) of the umbilical artery throughout pregnancy. Different letters above the graph points indicate significant differences.

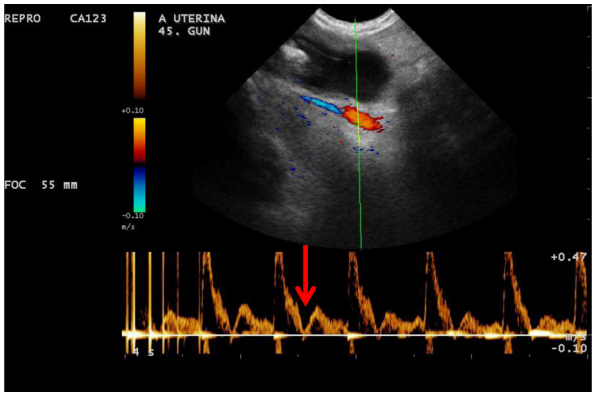


Fig. 4. Pathological diastolic notch (red arrow) in the uterine artery waveform of a pregnant ewe diagnosed with embryonic resorption on Day 45 of pregnancy. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

for the fetal aorta. Galan et al. [19] reported that the PI of the fetal aorta was higher in ewes with experimentally induced IUGR. The mean PI of the fetal aorta in fetuses with IUGR was 1.5 on Day 105 of pregnancy, compared with 1.1 in healthy fetuses. In the present study, the mean fetal aorta PI was similar to that of healthy fetuses in the aforementioned study by Galan et al. [19].

Serin et al. [3] reported that the umbilical artery PI increased from Days 40 to 55 of pregnancy and decreased significantly from Day 85 in pregnant goats. In our study, a decrease in the PI was detected from Days 60 to 120 of pregnancy. The umbilical artery PI and RI began to decrease toward delivery, as reported for pregnant bitches [20]. Similarly, the umbilical artery blood flow velocity increases with the progression of human pregnancy, and the resistance against blood flow thus decreases [21]. The umbilical artery RI on Days 83 and 98 of pregnancy was higher in the fetuses of

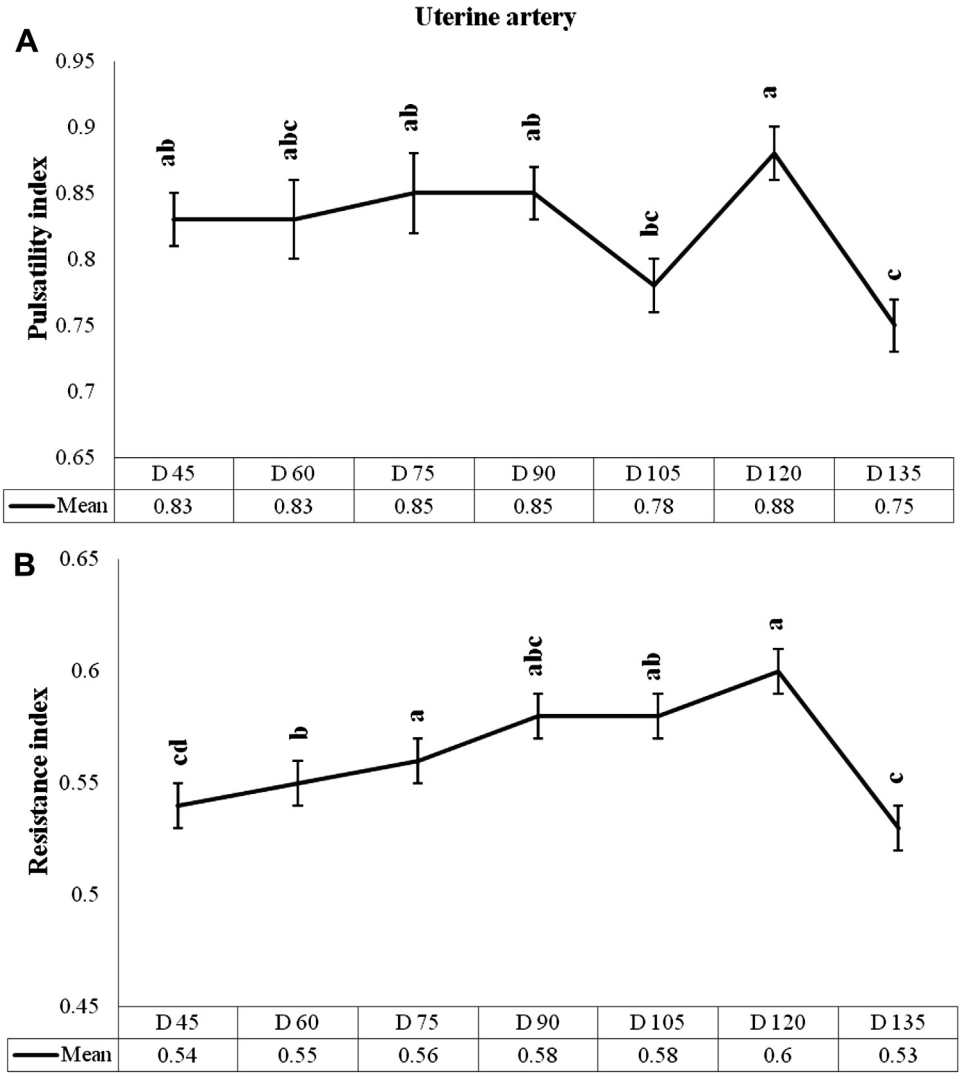


Fig. 5. Time-dependent changes in the pulsatility index (A) and resistance index (B) of the uterine artery throughout pregnancy. Different letters above the graph points indicate significant differences.

overnutritioned ewes in a study by Carr et al. [22]. There were no compromised pregnancies in our study, so we could not compare our results with those of Carr et al. [22]. The effect of single or twin pregnancy on Doppler USG findings in the fetal aorta was not significant in this study, in accordance with Serin et al. [3].

The Doppler velocity waveform of blood flow in the fetal vena cava can provide information about the existence of fetal arrhythmia, the status of fetal atrial filling, and the extent of any fetal hemodynamic damage in compromised pregnancies [23]. The PI in the fetal vena cava increases in cases of fetal hypoxia with the aim of compensation [24]. When the raw data of the present study were investigated, no such results were seen.

Three major pathologies are defined in the uterine blood flow of pregnant women. These are high resistance, bilateral notching, and unilateral notching [25]. Notching in the uterine artery waveform is normal in early human pregnancy. However, it should disappear from week 24 [25,26]. In our literature search, we found no publication regarding the normal disappearance time of uterine notching in pregnant ewes. However, uterine notching was only seen in one ewe diagnosed with embryonic resorption on Day 45 of pregnancy in this study. Two ewes were diagnosed with embryonic resorption. Only the uterine artery Doppler USG findings of these ewes could be recorded. The uterine artery PI and RI in the two ewes (1.76 and 0.79, respectively, in one and 2.06 and 0.81, respectively, in the other) were much higher than the overall mean uterine artery PI and RI (0.82 $\pm$ 0.02 and 0.54 $\pm$ 0.01, respectively). Uterine artery Doppler USG examination on Days 40 to 45 of pregnancy in ewes might be useful to evaluate the risk of embryonic resorption. However, further studies with many more compromised pregnancies are needed to obtain statistically meaningful results.

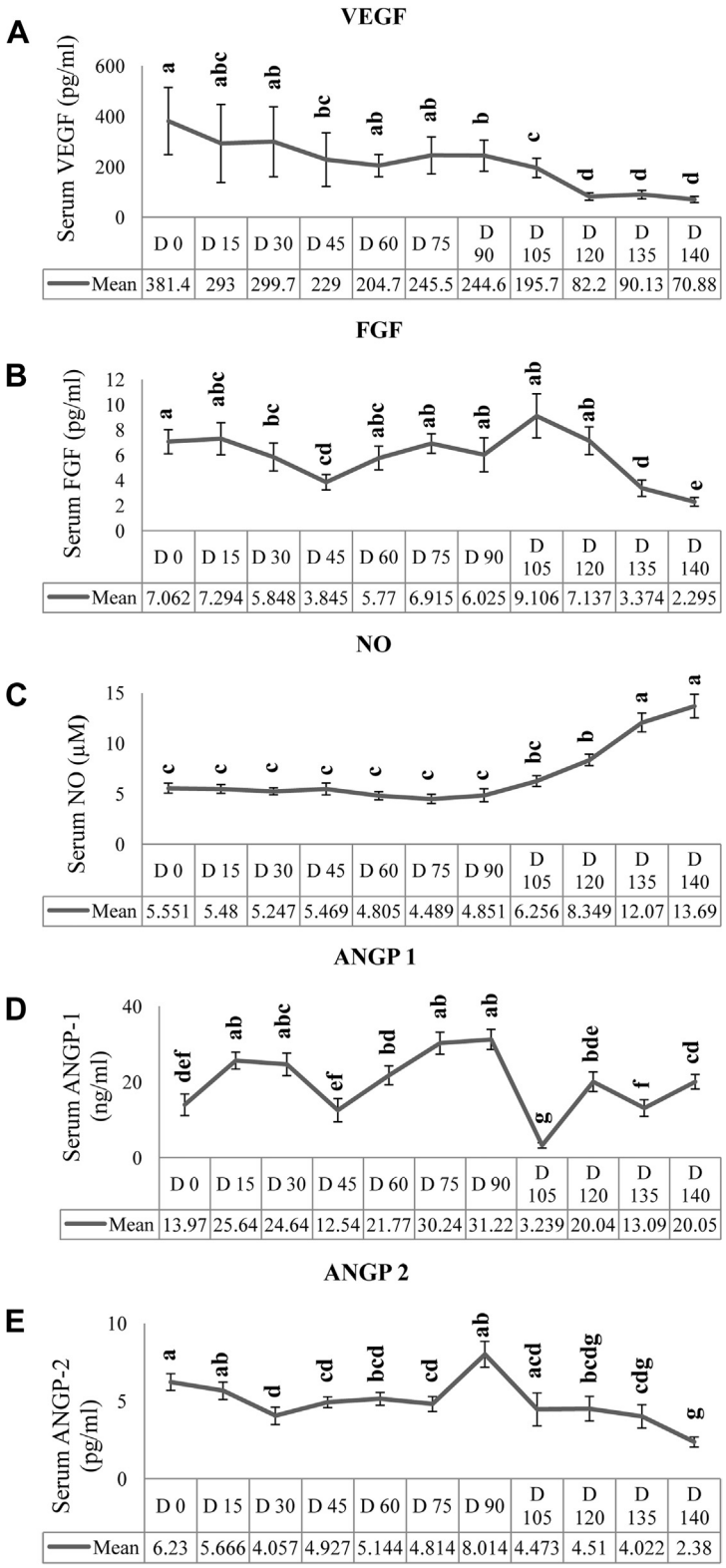


Fig. 6. Time-dependent changes in maternal serum angiogenic factor concentrations throughout pregnancy. (A) VEGF; (B) FGF; (C) NO; (D) ANGP-1; (E) ANGP-2. Different letters above the graph points indicate significant differences.

Although there are many angiogenic factors acting in fetal development, VEGF is one of the most effective factors and is responsible for placental and fetal development [27]. In women, the maternal serum VEGF concentration increases with the progression of pregnancy [28]. However, the serum VEGF concentration decreases from Days 20 to 69 in pregnant ewes, and the level is the same as that in nonpregnant ewes after Day 70 [27]. In the present study, a decrease was seen throughout pregnancy. However, our results could not be compared with nonpregnant ewes. VEGF binds to a protein named soluble fms-like tyrosine kinase 1 (sFlt-1), which controls the entry of VEGF into the target cell [29]. However, Vonnahme et al. [27] could not detect the sFlt-1 protein in sheep blood samples. The difference between women and ewes in the progression of VEGF throughout pregnancy might arise from the unknown relationship between VEGF and the sFlt-1 protein in sheep.

In previous studies [30,31], FGF expression was detected in sheep placental and uterine tissues only during early pregnancy, and thus, the researchers reported that FGF might be responsible for the onset of angiogenesis and uterine cell proliferation. In the present study, FGF was in measurable levels in maternal serum not only in early pregnancy but also throughout the entire pregnancy. Moreover, a significant decrease was determined at Day 135 of pregnancy. This may be associated with the potential role of FGF in lambing. Nevertheless, this should be supported with further studies.

Grazul-Bilska et al. [30] identified some uterine angiogenic factors in sheep in early pregnancy. According to their study, the messenger RNA expression of ANGP-1 in the uterus increased 2.5-fold from Days 21 to 30 of pregnancy. In the present study, the maternal serum ANGP-1 concentration increased from Days 15 to 30 of pregnancy, consistent with other studies, but then returned to baseline. VEGF induces vasculogenesis and angiogenesis. ANGP-1 helps in the maturation of vascular networks. ANGP-2 stops the activity of ANGP-1 and enables the formation of new branches in the network. Any failure in this ternary system would cause disruption in placentation [32,33]. ANGP-1 levels followed a fluctuating course throughout pregnancy in the present study. In particular, a very significant decrease in the serum ANGP-1 concentration was observed on Day 105. The increased ANGP-2 level on Day 90 might be the reason for the ANGP-1 increment on Day 105.

NO is a vasorelaxant that originates from the endothelium. It has a potent role in the regulation of placental and fetal blood flow and on the transport of nutrients and oxygen from the mother to the fetus [32]. Yang et al. [34] reported that the maternal serum NO concentration was higher in nonpregnant than pregnant ewes. In the present study, the maternal serum NO concentration was similar between nonpregnant and pregnant ewes until Day 135 of pregnancy. The NO concentration began to increase after that, in accordance with the findings of Yang et al. [32]. The decrease in the uterine, and umbilical artery RI at the end of pregnancy might be related to the increase in the serum NO concentration.

4.1. Conclusions

Doppler USG is a noninvasive procedure for the follow-up of pregnancy in ewes. The significant decrease in the PI and RI of the umbilical artery after Day 75 might be useful for evaluating the pregnancy status. One of the most interesting Doppler USG findings in the present study was the significant decrease in the PI and RI of the uterine arteries from Day 135. This finding might also be useful for determining the delivery time in practice. The elevated PI and RI of the uterine arteries in two ewes with embryonic resorption should be investigated with further studies. The significant increase in the maternal serum NO concentration on Day 135 suggests that NO might have a participation on the preparatory mechanism of lambing. The results of this study illustrate the progressive changes in the Doppler USG findings of the fetomaternal vessels and maternal serum angiogenic factors that occur throughout ewe pregnancy. The umbilical and uterine artery Doppler USG findings and maternal serum NO concentration may be important parameters for evaluation of the course of pregnancy in ewes. The results of this study should be compared with those of future studies that include compromised pregnancies and nonpregnant ewes.

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Competing Interests

The authors declare that they have no conflicts of interests.

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