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

Özge Turna Yılmaz\*, Mehmet Can Gündüz, Gamze Evkuran Dal, Melih Uçmak, Zeynep Günay Uçmak, Esra Karaçam, Güven Kaşıkçı, Mehmet Ragıp 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](mailto:turnao@istanbul.edu.tr) (Ö.T. Yılmaz).

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  $-20^{\circ}\text{C}$  until analysis. Concentrations of VEGF, bFGF, ANG-1, and ANG-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 ANG-1, 4.1 pg/mL for ANG-2, and 2.5  $\mu\text{M}$  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  $\pm$  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.

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