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Pulse wave Doppler ultrasound of umbilical cord in experimentally induced pregnancy toxemia in sheep

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ABSTRACT

Contrary to its widespread use in human cases, the use of Doppler ultrasonography is only recently becoming prevalent in farm animals. This study aimed to determine the effects of maternal metabolic and clinical changes on fetal hemodynamics during pregnancy toxemia with the doppler examination of umbilical cord. In the study twenty ewes with a single healthy fetus were included in the study. At the end of the 120th day of pregnancy, 20 single-bearing pregnant ewes were randomly categorized into two groups. Ewes in the control group were fed to meet all nutritional requirements. On the other contrary, the experimental ewes were fed to meet equivalent to 50 % of the daily needs and then fasted for 96 h. Doppler ultrasonographic examinations of umbilical cord were performed once every two days and once a day during fasting. Beta hydroxybutyric acid (BHBA) concentration was measured by taking blood from sheep on examination days. Pulse systolic velocity (PSV), end diastolic velocity (EDV), PSV/EDV, pulsatility index (PI), resistance index (RI), and fetal heart rate (FHR) as well as BHBA values and how those parameters has changed over time (time by treatment effect) due to energy deprivation during pregnancy were evaluated using repeated measure analysis of variance. No clinical signs were observed in both toxemia and control groups during restricted feeding. BHBA concentration increased and there was a significant time, time by treatment and main effect of treatment effect between groups. No significant main effect of treatment and time by treatment interaction was observed in the changes of PI, RI, FHR, and systolic/diastolic velocity values over time in both groups. FHR was reduced over time, and there was a significant time effect in FHR in both groups. Although doppler indices didn't increase, both PSV and EDV values increased significantly in the pregnancy toxemia group compared with the controls (Time $P = 0.03$, time by treatment interaction $P < 0.05$) and the main effect of treatment $P < 0.05$). The marked increase in blood velocities (PSV and EDV) in the umbilical cord is probably due to the compensatory functioning for excessive energy deprivation of the fetus. Therefore, PSV and EDV might be a valuable indicator for evaluating the fetus's health status during the management of the PT.

1. Introduction

Small ruminants, particularly native breed kinds, play a significant role to the livelihoods of a considerable part of the human population in the tropics from socio-economic aspects (Gebremedhin et al., 2013; Roudbar et al., 2017). Thus, combined trials with emphasis on administration, nutrition and genetic progress to improve animal outputs are of decisive significance (McGrath et al., 2018; Mohamadipoor Saadatabadi et al., 2021). Economical and biological efficiency of sheep production enterprises generally improves by increasing productivity and reproductive performance of ewes (Gazzarin and El Benni, 2020; Roudbar et al., 2018).

Pregnancy toxemia (PT) is a metabolic disorder that occurs during the final stage of gestation when energy requirements increase by 70 %–80 %. The disease commonly affects small ruminants, and the incidence can be as high as 20 % in herds (Mongini and Van Saun, 2023). Diagnosis

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is usually based on clinical signs and biochemical parameters, such as maternal pH, minerals, glucose, beta-hydroxybutyric acid (BHBA), and non-esterified fatty acid concentrations. The main therapeutic intervention is glucose supplementation. Although PT is a common disease, there is still no effective and definitive treatment for it especially if it is not diagnosed at an early stage. Therefore, disease mortality may be up to 80 %. (Bayne, 2023; Ji et al., 2023). Pregnant ewes may die even during treatment, and even if they recover, premature births, abortions, and stillbirths may occur. If the ewe does not respond to treatment, delivery can be stimulated, or an emergency cesarean section can be performed to save the offspring. The clinical approach in PT management usually is determined with the use of the abovementioned maternal markers as prognostic indicators for diagnostic purposes (Anteveli et al., 2023; Brozos et al., 2011; Lima et al., 2012). As the examination of the fetus of ewes with the disease is not performed, there may be loss of offspring even if the ewe survives. Thus, it is crucial to assess the fetal health status during treatment.

PT is a complicated pregnancy issue that starts with energy deficiency and is caused by hypoglycemia (Jeffrey and Higgins, 1992; Kabakci et al., 2003). Doppler ultrasonography is widely used in women to monitor the fetus in risky pregnancy cases (Alfirevic et al., 2017). Additionally, because pregnant sheep are used as animal models for women, there is a substantial amount of investigation on hemodynamic changes in sheep fetuses (Barry and Anthony, 2008). Contrary to its widespread use in human cases, Doppler ultrasonography is only recently becoming prevalent in farm animals. Although there are numerous study has been made with B-mode ultrasonography (Hu et al., 2023; Setyani et al., 2021), only limited studies on the use of Doppler ultrasonography in the veterinary field, particularly in farm animals (Bartlewski, 2019; Petridis et al., 2017; Ramírez-González et al., 2023). In studies conducted in the veterinary field, it has been revealed that the changes in the umbilical artery can be examined from the 70th day of pregnancy in sheep (Stankiewicz et al., 2020). The physiological data obtained in these studies will be useful in evaluating the health status of the fetus during adverse conditions such as PT. However, more work is needed to determine critical threshold levels of the physiological hemodynamics of the fetus. As for pregnancy pathologies in sheep, doppler studies are scarce. In the study of Barbagianni (2016), fetal hemodynamic changes in the umbilical cord were investigated in experimentally induced subclinical PT in sheep. Treatment success increases when metabolic changes are followed and detected at an early stage before clinical symptoms appear in pregnancy toxemia. Treatment success increases when metabolic changes are followed and detected at an early stage before clinical symptoms occur in pregnancy toxemia. However, treatment success is variable, especially when symptoms of the middle or terminal stage of the disease are observed (Abreu-Palermo et al., 2021). In case of failure in medical treatment, if the pregnancy is not terminated by cesarean section or medical intervention, the disease may result in the death of both the mother and the fetus (Simpson et al., 2019). Therefore, evaluation of the health status of the fetus, especially in the advanced stages of the disease, can provide the right decision to be made during treatment and prevent losses.

This study aimed to determine changes in maternal metabolic, clinical, and fetal hemodynamics in ewes either exposed or not to nutrient restriction to induce pregnancy toxemia with clinical observation and the doppler examination of the umbilical cord.

2. Materials and methods

The study protocol was approved by the Animal Experiments Local Ethics Committee of Sivas Cumhuriyet University (Approval number 324; Approval date, 15.05.2020). The study was conducted in a commercial sheep farm located in Sivas region of Türkiye.

2.1. Animals

The study was performed on 50 healthy Kangal breed ewes 2–4 years of age, given birth at least once, without any disease, and had no medication in the last 2 months. The animals included in the study were estrus synchronized during the mating season. The synchronization scheme is shown in Fig. 1. Two days after the sponge removal, the ram was introduced to ewes for 1 h each in the morning and evening, and the mated animals were taken to different compartments and separated from the rams. On the 25th (±2) day after ram introduction, pregnancy examination was performed using transrectal ultrasonography, and twin and single-bearing ewes were determined. Transabdominal ultrasonographic pregnancy examination was performed on day 60 (±2) to confirm the pregnancies. Finally, all animals were subjected to pregnancy examination transabdominally on day 110 (±2). Twenty animals with a single healthy fetus were included in the study. Pregnancy examinations were achieved using a 5 MHz linear probe (E.I Medical Ibex Lite). The animals included in the study were weighed, and their body weights and body condition scores were determined, as described in Ferguson et al. (1994). Four weeks before the delivery (120th day of gestation), the animals were randomly categorized into two equal groups, i.e., PT and control, and placed in individual compartments. The inguinal and partial abdominal regions of the ewes were shaved to allow ultrasonographic evaluation before starting the restricted feeding intervention.

2.2. Experimental procedure

In the first 120 days of gestation, all ewes were fed with a mixture of meadow hay and wheat straw ad libitum and supplemented with 250 g/day/head of commercial concentrate (12 % CP and 2750 kcal of ME kg^{-1}) according to the recommendation of Council, N.R (2007). The animals had free access to clean water and salt licks. At the end of the 120th day (considered as day 0 of the experimental period), 20 single-bearing pregnant ewes were randomly categorized into two groups. Ewes in the control group ($n = 10$) were fed to meet the nutritional requirements of single-bearing pregnant ewes in late pregnancy until their parturition (Council, N.R, 2007). On the other contrary, to create a pregnancy toxemia-inducing environment, the experimental ewes (PT group n = 10) were fed with a mixture of meadow hay and wheat straw diet (equivalent to 50 % of the daily needs, 1.52 Mcal metabolizable energy (Table 1) for 20 days and then fasted for 96 h. After 96 h of fasting, the study was terminated. The contents and chemical compositions of the diets given to the ewes during the experimental period are presented in Table 1.

Animals in the PT group were fasted from day 140 (±2) to determine the effects of terminal signs of toxemia on fetal circulation. Fasting was ended on day 144.

2.3. Chemical analysis

Dry matter, crude ash, ether extract, and crude protein were determined according to the procedure of AOAC (1995). The neutral detergent fiber was determined using sodium sulfite and heat-stable alpha-amylase, as described by Mertens et al. (2002). Acid detergent fiber (ADF) was determined according to the procedure of AOAC method 973.18 (AOAC, 1995). NDF and ADF were analyzed using the fiber

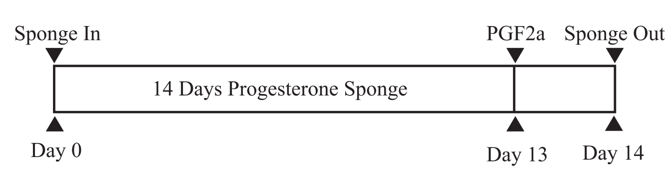

Fig. 1. Estrus synchronization scheme.

Table 1
Ingredients and chemical composition of the diets given in the experimental period.

	Groups	
Items	Control	Experimental
Ingredients, % DM		
Meadow hay	48	20
Wheat straw	12	80
Corn	16.5	–
Barley	10.5	–
Wheat bran	4	–
Sunflower meal, %36	3.5	–
Molasses	4	–
Salt	0.4	–
$CaCO_3$	0.7	–
Mineral–vitamin premix[1]	0.4	–
Chemical composition, % DM		
Dry matter	90.6	94.5
Crude ash	7.7	8.6
Crude protein	11.5	5.2
Ether extract	2.7	1.3
Neutral detergent fiber (aNDFom)	50.9	65.7
Acid detergent fiber (ADF)	31.4	42.0
Metabolizable energy[2](Mcal/kg DM)	2.55	1.52

[1] Each kg contained 50,000 mg Mn, 50,000 mg Fe, 50,000 mg Zn, 10,000 mg Cu, 150 mg Co, 150 mg Se, 800 mg I, 8,000,000 IU vitamin A, 2,000,000 IU vitamin D3, and 20,000 mg vitamin E.
[2] Calculated according to NRC (2007).

analyzer (Ankom 200, Ankom Technology Corp., USA) and expressed exclusive of residual ash.

2.4. Doppler ultrasonography

Ultrasonographic examinations were performed once every two days until fasting and once a day during fasting after the morning feeding (within 2–4 h). The examinations were performed without sedating the animals. Ultrasonographic examinations were done in the quadrupedal position. The examinations were always performed by the same experienced and trained veterinarian, with appropriate restrictions and handling. Briefly, the examination was performed in the inguinal and abdominal regions as previously described (Serin et al., 2010) using an ultrasound scanner (Mindray DC-N3 Vet, Shenzhen Mindray Animal Medical Technology, China) equipped with a 2 MHz convex probe. Doppler-derived blood flow velocity waveforms of the umbilical artery (UA) were obtained from the free loop of the umbilical cord. Color Doppler was used to identify arterial and venous vessels in the umbilical cord (Fig. 2). At least three consecutive systolic peaks with similar velocity and amplitude were recorded. Measurements were not recorded during maternal and fetal movements. Umbilical cord Peak systolic velocity (PSV), end-diastolic velocity (EDV), PSV/EDV ratio (S/D), resistance index [RI = (PSV − EDV)/PSV], pulsatility index [PI = (PSV–EDV)/mean velocity], and fetal heart rate (FHR) values were automatically calculated for each waveform. The insonation angle during the examination was kept as close as possible to 0 degrees. After the animals were restrained, the examinations took <5 min. Measurements with pulsed-wave Doppler took <30 s. After the animal was restrained, in case of stress (excessive movement, refusal to allow the examination, etc.) or if the examination could not be performed within

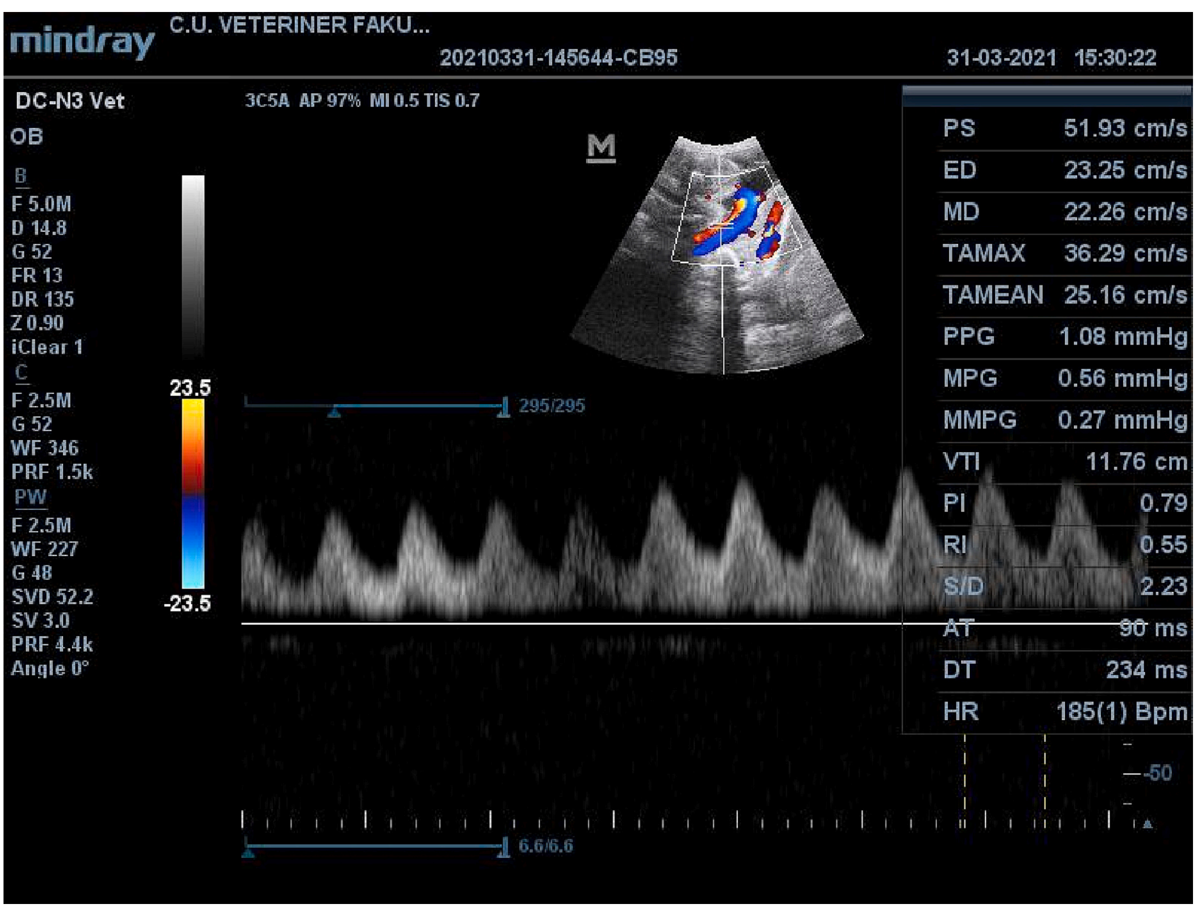

Fig. 2. Pulsed doppler examination and identification of umbilical cord with color doppler.

10 min, the examination was terminated and performed a few hours later.

2.5. Blood sampling and analysis

Blood samples from the vena jugularis every 48 h from day 120 to day 140 were collected with an 18-gauge sterile syringe. Blood samples were always obtained in the morning before the first feeding. On day 140, the animals were fasted, and blood was collected every 24 h. BHBA concentrations were measured in the samples with an electronic handheld BHBA meter (Precision Xceed, Abbott Diabetes Care Ltd.) (Pichler et al., 2014).

2.6. Statistical analysis

Statistical analysis of the study data was performed using the SPPS 25 (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.) statistical package program. Student's *t*-test was used to evaluate age, body condition score, and live body weight data. Before analysis, normality and homogeneity were verified using the Shapiro–Wilk and Levene tests, respectively. PSV, EDV, PSV/EDV, PI, RI, and FHR as well as BHBA concentrations were compared between the groups via analysis of variance for repeated measures using a mixed-model procedure for treatment, time, and treatment-by-time interaction. The conformity of the data for repeated measures analysis of variance was evaluated using Mauchly's sphericity test. The Bonferroni correction was applied for multiple comparisons.

3. Results

The mean body weight was 65 ± 4.5 kg (BCS 3.5 ± 0.5) in the PT group and 64 ± 3.2 kg (BCS 3.5 ± 0.5) in the control group. The mean age of the animals in the PT and control groups was 3 ± 1 years. No statistical difference was observed in body weight, BCS, or age between the two groups.

No end-diastolic block, reverse flow, or end-systolic notching were observed during Doppler ultrasonography. No clinical signs were noted in the control group during the study. No clinical signs were observed in both toxemia or control groups during restricted feeding (Between days 120 and 140). On day 2 of fasting, four animals had difficulty in standing up and experienced muscle tremors. On day 3 of the treatment, one animal showed signs of star counting. Lateral recumbency, convulsions, self-auscultation, and nystagmus were seen in three animals on day 4 of fasting.

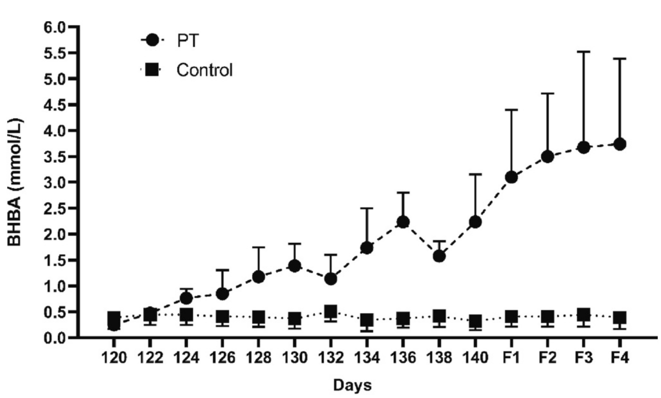

Fig. 3. Changes in serum BHBA levels in single-bearing 20 healthy ewes with adequate (control $n = 10$) restricted feeding (PT n = 10) from 120 days of gestation and fasted after 140 days of gestation. There was an effect of treatment ($P = 0.01$), time ($P < 0.01$) and treatment by time interaction (P < 0.01) in BHBA concentrations.
F: Fasting.

Serum BHBA concentration, PSV, EDV, PSV/EDV, (S/D), PI, RI, and FHR are shown in Figs. 1–7. There was an effect of treatment ($P = 0.01$), time ($P < 0.01$), and treatment by time interaction (P < 0.01) on BHBA concentrations in the PT group. ($P < 0.05$). As for the control group, there was no time and main effect of treatment in BHBA concentration ($P = 0.8$). The concentration started to increase after the onset of restricted feeding and soon exceeded 0.5 mmol/L. It reached the critical concentration of 1.6 mmol/L at the end of 1 week and rose above 3.5 mmol/L at the beginning of the fasting intervention (Fig. 3).

There was no significant main effect of treatment and time by treatment effect in the changes of PI, RI, FHR, and S/D values ($P > 0.05$) (Fig. 4-7). Although clinical signs were observed in the toxemia group, the change in these values over time did not differ between the groups excluding FHR (Time by treatment effect) (P > 0.05). FHR significantly reduced over time in both groups (Time effect $P = 0.04$). PSV and EDV values changed significantly over time (Time effect) $P = 0.03$ (Figs. 8–9). Both PSV and EDV values increased significantly in the PT group (Main effect of treatment) (P < 0.05). Also, there was significant time by treatment interaction (P < 0.05) in PSV and EDV between groups. The first significant increase in PSV and EDV was observed on the first day of fasting, and these values decreased after the 2nd day of fasting.

4. Discussion

Our data clearly showed that study methods were successful in initiating pregnancy toxemia in the toxemia group, while no evidence of pregnancy toxemia was observed in the control group. BHBA concentration increased significantly in sheep in the PT group with restricted feeding and fasting. Although PI, RI, S/D, and FHR were similar between the groups in UA Doppler measurements, PSV and EDV values increased significantly in PT ewes at the beginning of fasting intervention when energy deficiency became evident.

In previous studies in which experimental PT was induced, restricted feeding and fasting for 72 h have been commonly used (González et al., 2011; Hefnawy et al., 2010). However, considering the natural occurrence of the pathology of PT, the disease starts with an increase in energy need and decreased feed intake due to the effect of the growing fetus (Brozos et al., 2011). We, therefore, first applied restricted feeding and then fasting, considering natural PT progression. As single-bearing ewes and a fat-tailed breed were used in the study, the fasting period was determined to be 96 h, unlike previous studies.

BHBA is a widely used indicator of negative energy balance (Kalyesubula et al., 2019; Zou et al., 2021). The serum BHBA concentration was 0.5 mmol/L in both groups at the first measurement. The concentration increased gradually when restricted feeding was started on day 120 and exceeded 3.5 mmol/L in the restricted feeding group. In the control group, the serum BHBA concentration remained <0.5 mmol/L on average during the same period. Based on the BHBA concentrations, it was concluded that the experimental procedure induced a progressive energy deficiency similar to the mechanism of naturally occurring PT. The clinical signs of PT observed in animals in the PT group also proved that restricted feeding and fasting intervention caused the condition.

FHR variability has been widely used clinically to assess fetal health (Ardakani et al., 2022; Cruz-Aleixo et al., 2021; Ghesquière et al., 2022). There is a significant negative correlation between FHR and fetal age. FHR decrease as fetal age increases (Ardakani et al., 2022). In our study, the FHR bpm was 170–200 on day 120. At the end of the fasting period, the FHR level was 110–150 bpm in all animals (Fig. 4). The change in FHR over time significantly differed in both groups (Time effect). However, despite the unexpected observation of clinical signs of PT, there was no difference in FHR between the two groups (Main effect of treatment and time by treatment effect).

In the veterinary literature, there are limited studies on the use of Doppler ultrasonography to assess fetal health (Petridis et al., 2017). In the last trimester of intrauterine life, PSV and EDV values markedly increase as umbilical vascular resistance decreases and umbilical blood

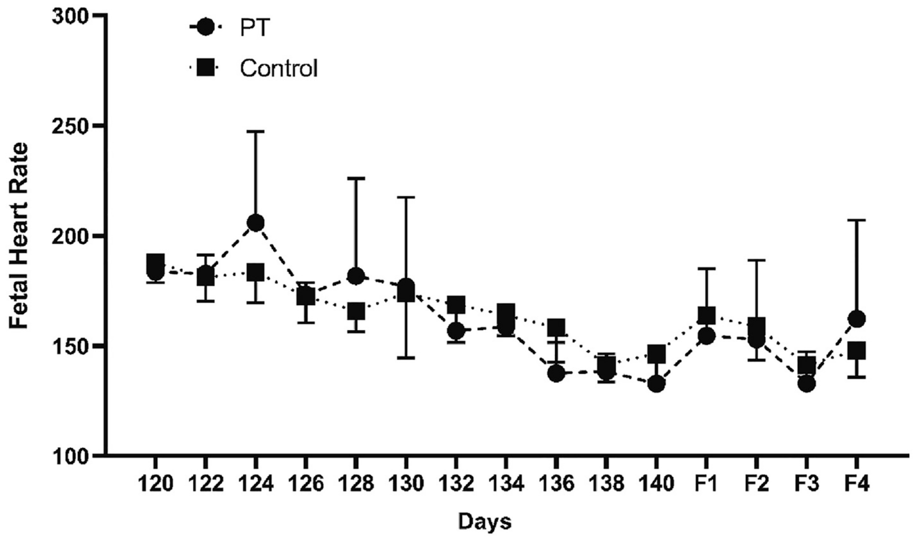

Fig. 4. Changes in fetal heart rate obtained using umbilical artery Doppler measurements in single-bearing 20 healthy ewes with adequate (Control n = 10) and restricted feeding (PT n = 10) from 120 days of gestation and fasted after 140 days of gestation. Fetal heart rate significantly reduced over time in both groups (Time effect $P = 0.04$). There was not a significant main effect of treatment ($P > 0.05$) and Time by treatment ($P > 0.05$) effect in fetal heart rate. F: Fasting.

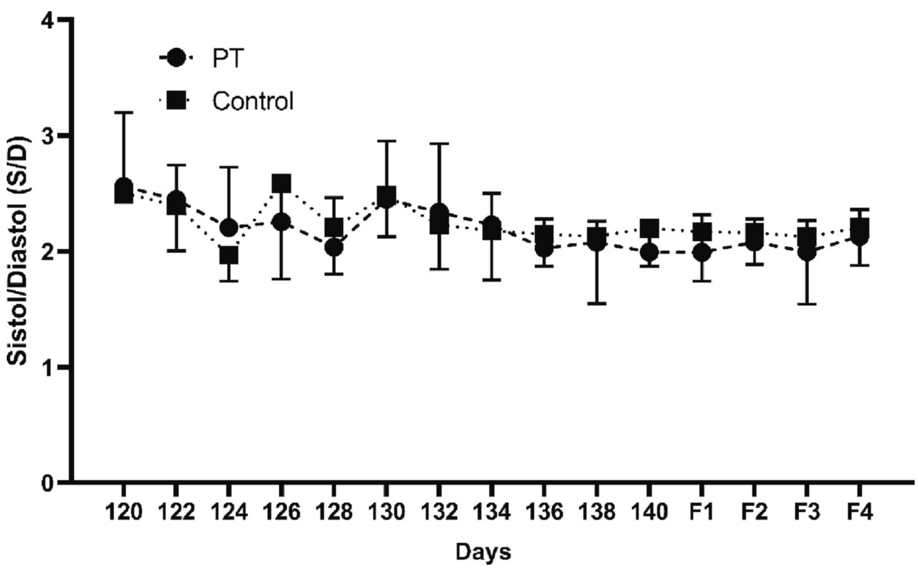

Fig. 5. Changes in S/D ratio obtained using umbilical artery Doppler measurements in 20 single-bearing healthy ewes with adequate (Control n = 10) and restricted feeding (PT n = 10) from 120 days of gestation and starved after 140 days of gestation. There was no effect of treatment ($P > 0.05$), time ($P > 0.05$) and treatment by time interaction ($P > 0.05$). F: Fasting.

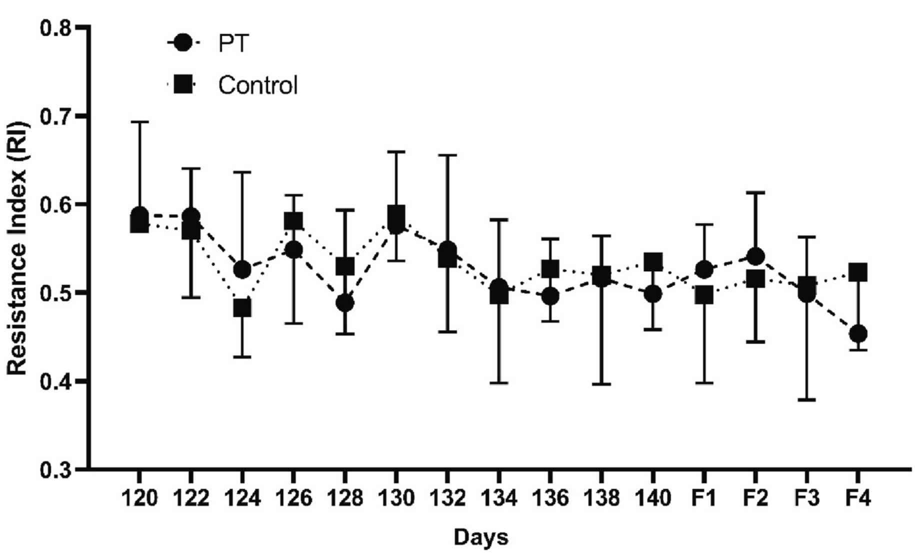

Fig. 6. RI change obtained using umbilical artery Doppler measurements in single-bearing 20 healthy ewes with adequate (Control n = 10) restricted feeding (PT n = 10) from 120 days of gestation and starved after 140 days of gestation. There was no effect of treatment ($P > 0.05$), time ($P > 0.05$) and treatment by time interaction ($P > 0.05$).
F: Fasting.

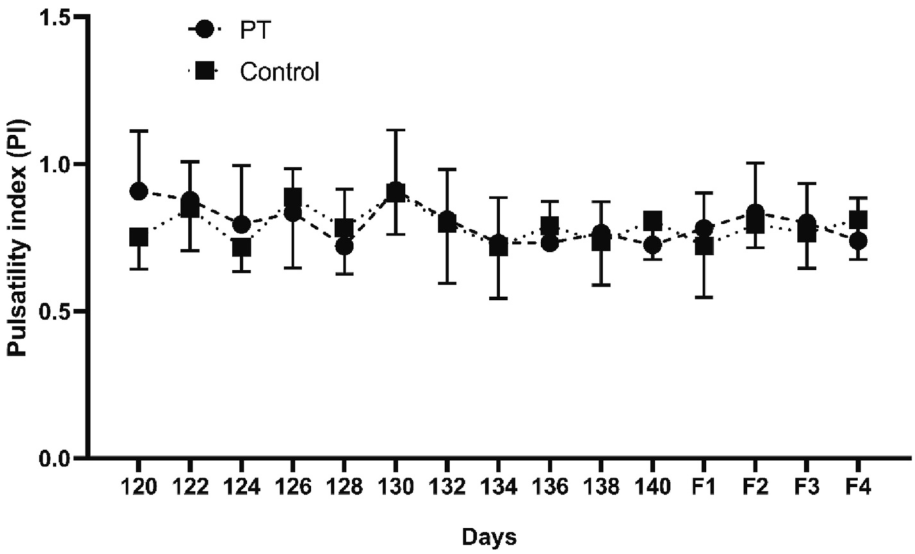

Fig. 7. PI change obtained using serum umbilical artery Doppler measurements in single-bearing 20 healthy ewes with adequate (Control n = 10) and restricted feeding (n = 10) from 120 days of gestation and fasted after 140 days of gestation. There was no effect of treatment (P > 0.05), time (P > 0.05) and treatment by time interaction (P > 0.05).
F: Fasting.

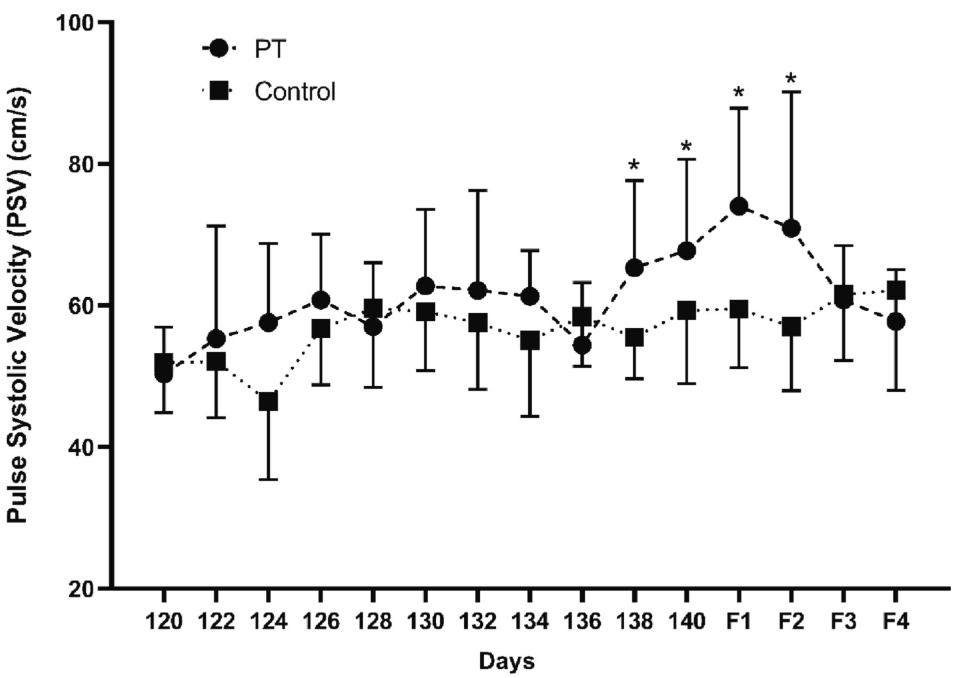

Fig. 8. Changes in PSV level obtained using serum umbilical artery Doppler measurements in single-bearing 20 healthy ewes with adequate (Control n = 10) restricted feeding (PT n = 10) from 120 days of gestation and starved after 140 days of gestation. There was a time ($P = 0.03$) main effect of treatment ($P < 0.05$) and time by treatment interaction (P < 0.05).
F: Fasting.
*$P < 0.05$.

flow velocity increases to meet fetal requirements (Almeida Lima Veiga et al., 2018; Brzozowska et al., 2022). On the contrary, PI and RI decrease during the last trimester of gestation compared with the second trimester in the assessment of fetuses (Brzozowska et al., 2022; Serin et al., 2010). However, there was no significant change in PI and RI levels in the last 7–8 weeks of gestation (Brzozowska et al., 2022). Similar to previous studies, in the present study, there was no significant change in PI and RI values after 120 days of pregnancy in both groups. However, despite the severe negative energy balance, there was no difference in Doppler indices between the two groups. Barbagianni et al. (2015) found that there was a significant decrease in Doppler indices in twin-bearing ewes fed with low-energy ration in the last trimester of pregnancy and that this decrease was proportional to the level of energy deficiency and the severity of PT. However, Abd-El-Aal et al. (2009) observed in their study that Doppler indices in UA measurements obtained in sheep fasted for a short time (12h) were comparable to those of normally fed sheep. Similarly, no significant change was observed in Doppler indices in UA Doppler measurements in rabbits with experimental ketosis (Ural and Erdogan, 2018).

Although there was no change in PI and RI levels, PSV and EDV values were significantly increased in animals with PT. PI and RI values are obtained with proportional calculation using PSV and EDV values. Accordingly, although PI and RI are useful indicators for hemodynamic evaluation in many diseases, since it is a proportional calculation, they may not be useful in some cases where PSV and EDV function simultaneously (Diniz et al., 2008; Rosenberg et al., 1985). For example, PSV is preferred for the assessment of the fetal mid-cerebral artery in women during pregnancy instead of PI and RI (Brock et al., 2020; Tollenaar et al., 2019). In our study, RI and PI values did not differ between the groups during the study period, whereas PSV and EDV values increased significantly in the last days of restricted feeding and the first days of fasting when energy deprivation reached its peak. As fasting continued, PSV and EDV started to decline from the peak value and decreased to a level similar to that of the control group. In a study by Mari et al. (2007),

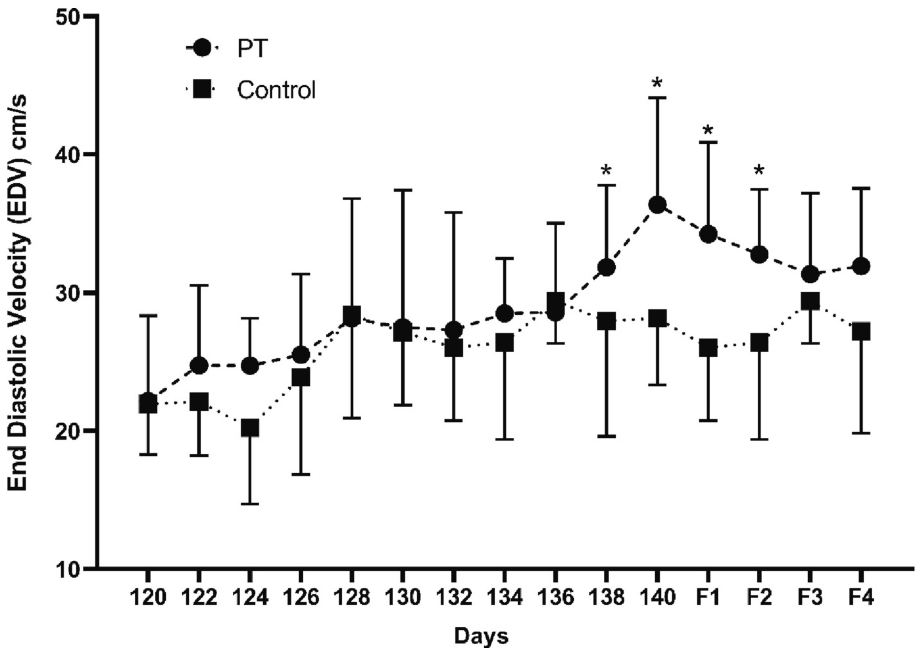

Fig. 9. Changes in PSV level obtained using serum umbilical artery Doppler measurements in single-bearing 20 healthy ewes with adequate (Control n = 10) restricted feeding (PT n = 10) from 120 days of gestation and starved after 140 days of gestation. There was a time (P = 0.03) main effect of treatment (P < 0.05) and time by treatment interaction (P < 0.05).
F: Fasting.
*P < 0.05.

it was determined that the PSV value in the mid-cerebral artery first increased as the fetal anemia worsens and then decreased significantly, which is similar to our findings. In the same study, a rise in PSV followed by a fall resulted in fetal death. The umbilical cord is vital for the healthy development of the fetus. The fetal requirements are met by the umbilical cord until birth (Di Naro et al., 2001). The decrease in hemoglobin level due to fetal anemia and the increase in pCO2 level cause an increase in PSV in fetal arterial blood flow. Thereby, The fetus tries to compensate for the existing adverse condition by changing the blood volume and viscosity (Mari, 2000; Papantoniou et al., 2013; Picklesimer et al., 2007). The marked increase in PSV and EDV in sheep with PT is probably due to the compensatory functioning for excessive energy deprivation of the fetus (Troisi et al., 2018). A decline in PSV may predict decompensated fetus and fetal mortality. On the other hand, as in fetal anemia, a decrease in hemoglobin level and an increase in pCO2 level occur in fetuses of sheep with PT. In the investigation of Tharwat and Al-Sobayil (2014), it was observed that the hemoglobin level decreased significantly in the samples taken from the umbilical cord of the fetuses of the sheep with pregnancy toxemia, and the pCO2 level significantly increased when compared to the healthy fetuses. However, in order to better understand the importance of PSV and EDV values and their use for prognostic purposes, there is a need to investigate how these values change in naturally occurring pregnancy toxemia and how they affect treatment results, viability and development of lambs.

5. Conclusion

The evaluation of Doppler ultrasonography data, BHBA concentrations, and clinical signs revealed that while BHBA concentrations increased progressively, there was no change in PI and RI values, and no clinical signs were observed in animals during the same period. Therefore, It may not be possible to evaluate the condition of the fetus by examining the Doppler indexes(PI, RI, and S/D). On the other hand, PSV and EDV were significantly changed before the clinical signs were developed. As a result, BHBA is indeed a simple and excellent indicator of maternal energy deficiency. However, our data showed that PSV and EDV measurements from UA could be a better indicator of fetal health status and well-being.

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Declaration of Competing Interest

None.

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