https://doi.org/10.52973/rcfcv-e34451 Revista Científica, FCV-LUZ / Vol. XXXIV, rcfcv-e34451

Adipokine concentrations in sheep with experimental pregnancy toxemia. A randomized, controlled clinical trial

Concentraciones de adipocinas en ovejas con toxemia de gestación experimental. Un ensayo clínico controlado y aleatorizado

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ABSTRACT

Pregnancy toxemia (PT) is a metabolic disease of small ruminant that develops during the last period of pregnancy and can cause death. Considering the high mortality rate, early diagnosis of the disease is important to minimize economic loss. Thus, this study aimed to investigate the concentrations of some adipokines (Leptin, Apelin, Resistin) and some other biochemical parameters for their role in the diagnosis and prognosis of PT. Fifty Kangal ewes that were between the ages of 2–4 year-old, were used in the study. The animals included in the study were subjected to estrus synchronization. Rams were introduced to all ewes subjected to synchronization. Pregnancies were diagnosed with ultrasonography on the 25th, 60th, and 110th days (d) following matings. Sixteen ewes bearing twin fetuses, whose fetal viability continued, were included in the study after general examinations. The ewes were maintained under grazing conditions in the first 110 d of gestation. At the end of the 110th d, 16 twin-bearing pregnant ewes were randomly divided into two groups (Control and PT). Ewes in the control group were fed to meet the nutritional requirements. the experimental PT group ewes were fed with equivalent to 50% of the daily needs for 20 d (120–140) and then fasted for 72 hours (141–143). During study blood samples were taken via jugular vein every 5 d from the 120th d to the 140th d. BHBA, glucose, Leptin, Resistin, Insulin and Apelin were measured in the blood samples. Liver biopsy samples were collected twice from all ewes on the 120th and 143rd d. As a result, in group PT, BHBA and leptin concentrations have been increased significantly while glucose levels significantly reduced. Resistin, Insulin and Apelin concentration were similar in both groups. In conclusion, it was concluded that monitoring BHBA, glucose and leptin in PT may be useful in diagnosis and prognosis.

Key words: Pregnancy toxemia; apelin; leptin; insulin; adipokines; Kangal ewes

RESUMEN

La toxemia de la gestación (PT) es una enfermedad metabólica de los pequeños rumiantes que se desarrolla durante el último período de la gestación y puede provocar la muerte. Teniendo en cuenta la alta tasa de mortalidad, el diagnóstico precoz de la enfermedad es importante para minimizar las pérdidas económicas. Por lo tanto, este estudio tuvo como objetivo investigar las concentraciones de algunas adipocinas (Leptina, Apelina, Resistina) y algunos otros parámetros bioquímicos por su papel en el diagnóstico y pronóstico del PT. En el estudio se utilizaron 50 ovejas de la raza Kangal que tenían entre 2 y 4 años. Los animales incluidos en el estudio fueron sometidos a sincronización de estro. Se introdujeron carneros a todas las ovejas sometidas a sincronización. Las hembras gestantes fueron diagnosticadas mediante ecografía los días 25, 60 y 110 después del apareamiento. Se incluyeron en el estudio 16 ovejas con fetos gemelos, cuya viabilidad fetal continuaba, después de exámenes generales. Durante los primeros 110 días de gestación, las ovejas se mantuvieron en condiciones de pastoreo. Al final del día 110, las 16 ovejas preñadas con gemelos se dividieron aleatoriamente en dos grupos (Control y PT). Las ovejas del grupo de control fueron alimentadas para satisfacer los requerimientos nutricionales. Las ovejas del grupo PT experimental fueron alimentadas con el equivalente al 50% de las necesidades diarias durante 20 días y luego ayunaron durante 72 horas (141–143). Durante el estudio, se tomaron muestras de sangre a través de la vena yugular cada 5 días desde el día 120 hasta el día 140. En las muestras se midieron BHBA, glucosa, leptina, resistina, insulina y apelina. Se recogieron muestras de biopsia hepática dos veces de todas las ovejas los días 120 y 143. Como resultado, en el grupo PT, las concentraciones de BHBA y leptina aumentaron significativamente mientras que los niveles de glucosa se redujeron significativamente. Las concentraciones de Resistina, Insulina y Apelina fueron similares en ambos grupos. En conclusión, la monitorización de BHBA, glucosa y leptina en PT puede ser útil.

Palabras clave: Toxemia de la gestación; apelina; leptina; insulina; adipocinas; Kangal ovejas

Received: 14/05/2024 Accepted: 12/07/2024 Published: 13/10/2024

INTRODUCTION

Pregnancy toxemia (PT) is a metabolic disease that develops due to negative energy balance (NEB) in small ruminants during the last period of pregnancy and causes hyperketonemia and hypoglycemia. The frequency of occurrence in herds can reach up to 20%. Although PT is a well-known disease that was identified many years ago, it can result in death if not diagnosed and treated early [1]. Sheep (*Ovis aries*) and lamb deaths in sheep farming cause great economic losses [2, 3]. The mortality rate in affected animals can reach 80%. Furthermore, 40% of mothers may die even after or during medical treatment (fluid, electrolyte, glucose, and propylene glycol), and even if they recover, premature birth, miscarriage, and stillbirth may occur [1, 4].

Pregnancy is an important period during which sheep must consume nutrients in sufficient quantity and quality to maintain basic metabolism and also ensure fetal growth [4]. Nutritional deficiency observed in sheep in the last stages of pregnancy; Insufficient energy in the diet or reduced rumen capacity due to fetal growth are two important risk factors for PT. As a compensation mechanism, the mobilization of fats is aimed at meeting the energy needs necessary to maintain fetal development and meet the needs of the sheep [5].

Adipose tissue is considered an important part of the body because it works as an endocrine organ in addition to its contributions to energy metabolism. The adipokines it secretes play a role in many physiological processes of the body, such as nutrition, appetite, energy balance, insulin and glucose metabolism, lipid metabolism, blood pressure regulation and inflammation [6, 7]. The process, which first started with the discovery of leptin in 1994, continued with the discovery of adiponectin, resistin, visfatin, apelin, omentin, chemin, nesfatin and a number of other adipokines [8]. The Apelin (AP) receptor was discovered in 1993 [9]. It has been found that AP is expressed at high levels especially in the hypothalamus. The hypothalamus is an important part of the brain that can secrete various appetite-regulating factors. This suggests that it may play an important role in regulating nutrition. Various studies conducted in mammals have also shown that AP plays an active role in food intake [10]. Resistin (RETN) was discovered as an adipose tissue-specific hormone in 2001 [11]. Studies conducted on animals; It has revealed the relationship of RETN with obesity, metabolic syndrome and Type 2 diabetes [12]. Hyperglycemia and hyperinsulinemia stimulate RETN secretion. In studies conducted on humans; A strong relationship has been observed between RETN and insulin resistance, high blood sugar and increased blood insulin levels [13]. Leptin (LP), synthesized from cells in white adipose tissue, regulates food intake, body mass, reproductive function, and plays a role in fetal growth, pro-inflammatory immune responses, angiogenesis, and lipolysis [14].

Excessive mobilization of fats to meet energy needs contributes to the formation and exacerbation of PT. Considering the high mortality rate, early diagnosis of the disease is important to minimize economic loss. Diagnosis may be difficult due to the nonspecific symptoms that animals affected by PT show in the early stages [15, 16]. Routine measurement of biochemical parameters and their simultaneous evaluation with clinical examination is an important tool in the diagnosis of diseases [17]. Therefore, biomarkers that will enable rapid decision-making are needed to enable treatment of the disease. Thus, this study aimed to investigate the concentrations of LP, AP, RETN and some other biochemical parameters for their role in the diagnosis and prognosis of PT.

MATERIALS AND METHODS

The study was conducted in a commercial sheep farm located (39°48′15.8″N | 37°07′18.7″E) in Sivas region of Türkiye. The research was conducted between March and August of 2023, when sheep are in the natural anoestrus period in the northern hemisphere.

Animals

In this study, 50 female Kangal sheep breeds that were between the ages of 2–4-year-old, had given birth at least once, had not had any disease in the last 2 months, and had been vaccinated within the scope of preventive medicine activities were used. The sheep did not receive any medication for the treatment of any disease, and they were healthy according to clinical findings and anamnesis information. The animals included in the study were weighed (Pinar, PR-110, Türkiye) and their live weights and body condition scores were determined as described by Ferguson *et al.* [18].

The animals included in the study were subjected to estrus synchronization induction during the anoestrous period, as described by Kivrak *et al.* [19]. In summary, intravaginal progesterone-impregnated sponge (a white, 40 × 30 mm cylindrical polyurethane sponge containing 20 mg Chronolone flugestone acetate; Chronogest® CR, MSD, Türkiye) application was performed on days (d) 0 for synchronization purposes. On the 13th d after sponge application, a single dose of PGF2α analogue (263 μg Cloprostenol sodium equivalent to 250 μg Cloprostenol per ml; PGS®, Alke, Türkiye) was administered intramuscularly (IM) to all animals. One day after prostaglandin application, the intravaginal sponge was removed and 480 IU eCG (each mL of solution for injection contains 240 IU of equine chorionic gonadotropin hormone; Chronogest/PMSG®, MSD, Türkiye) was administered IM to all animals. 2 d after the sponge was removed, all animals were treated with a ram for 1 hour (h), morning and evening, and the mated animals were taken into a separate compartment and away from the rams. At 25th (±2) d after the mating of the ram, a pregnancy examination was performed using transrectal ultrasonography (5 MHz linear probe, E.I Medical Ibex Lite, USA) and twin pregnant animals were determined. In order to confirm the pregnancies, the pregnancy examination was repeated on 60th (±2) d with transabdominal ultrasonography to determine fetal viability. Finally, on d 110 (±2), all animals were examined transabdominally for pregnancy, and 16 animals with twin offspring, whose fetal viability continued, were included in the study after general examinations. Before the start of restricted feeding, the right ribs were partially shaved to allow biopsy.

Experimental Design

In the first 110 d of gestation, the ewes were maintained under grazing conditions between 08:00 and 17:00 on medium-quality pasture (average 1500 kg DM·year^{-1}·ha^{-1}). They had ad libitum access to water and mineral salt and were housed in a barn for the rest of the day. At the end of the 110th d, the ewes were examined by ultrasound for pregnancy, and 16 twin-bearing pregnant ewes were selected and by draw lot randomly divided into two groups (n=8). Ewes in the control group were fed to meet the nutritional requirements of twin-bearing pregnant ewes in late pregnancy (10.5% CP and 2.35 Mcal·kg^{-1} of ME on a dry matter basis) until their parturition [20]. To adapt ewes in the control group to the control diet, a 10-d step-up protocol was implemented, gradually increasing the concentrate levels from 0 to 46% between d 110 and 120, respectively (TABLE I). Following grazing,

the PT group did not receive concentrate feed (10.5% CP and 2.35 Mcal·kg^{-1} of ME on a dry matter basis); consequently, no adaptation protocol was implemented. On the other hand, to produce pregnancy toxemia–inducing environment, the experimental PT group ewes were fed with a mixture of meadow hay and wheat straw diet (equivalent to 50% of the daily needs, 5.3% CP 1.62 Mcal·kg^{-1} of ME on a dry matter basis; TABLE I) for 20 d (120–140) and then fasted for 72 h (141–143). After 72 h of fasting, the study was terminated. TABLE I presents the contents and chemical compositions of the diets provided to the ewes during the 110 to d 143.

TABLE I
Content and chemical composition of the ration given to the ewes in the pregnancy toxemia (PT) group (n=6), which started with restricted feeding on the 120th day of pregnancy and starved between the 140th and 143rd days, and the ewes in the control group (n=7), which were adequately fed throughout pregnancy, during the study

	Groups	
Items	**Control**	**Experimental**
Ingredients, DM %		
Wheat straw	7.4	20
Meadow hay	38.5	79
Barley	15.3	–
Corn	22.6	–
Wheat bran	4	–
Sunflower meal, 36 %	7.2	–
Molasses	4	–
Salt	0.25	0.25
$CaCO_3$	0.45	0.45
Mineral–vitamin premix[1]	0.30	0.30
Chemical composition, DM %		
Dry matter	89.6	92.5
Crude Ash	8.7	9.6
Crude Protein	10.5	5.3
Ether extract	2.4	1.3
Neutral detergent fiber (NDF)	44.6	68.7
Acid detergent fiber (ADF)	31.8	44.0
Metabolisable energy[2] (Mcal·kg^{-1} DM)	2.36	1.62

[1]:Each kg contained 50.000 mg Fe, 50.000 mg Mn, 50.000 mg Zn, 10.000 mg Cu, 800 mg I, 150 mg Co, 150 mg Se, 8.000.000 IU vitamin A, 2.000.000 IU vitamin D3, 20.000 mg vitamin E. [2]:Calculated according to (NRC, 2007)

Chemical analysis

The dry matter, crude ash, ether extract, and crude protein were analyzed following the Association of Official Agricultural Chemists (AOAC) procedure [21]. Neutral detergent fiber (NDF) was determined using sodium sulfite and heat-stable alpha-amylase, as detailed in Mertens (2002) [22]. Acid detergent fiber (ADF) was determined using the AOAC procedure [21]. The analysis of both NDF and ADF were determined using the fiber analyzer (Ankom 200, Ankom Technology Corp., USA) and expressed exclusive of residual ash.

Blood Sampling and Analysis

For biochemical analyses, blood samples were taken from the Vena jugularis with 18-gauge sterile syringe every 5 d from the 120th d to the 140th d. Blood samples were always taken in the morning before the first feeding. After the animals started to starve on the 140th d, blood was taken every 24 h. For serum biochemical analysis, blood samples taken into vacuum tubes were kept at +4°C for 30 min and then processed in a refrigerated centrifuge (Nüve NF 800, Nüve Laboratory & Sterilization Technology, Türkiye) at 1400 g. Sera were removed by centrifugation for 10 min, distributed into labeled microtubes, and stored at -80°C (Haier, DW-86L828S, China) until the day of measurements. β-Hydroxy Butyric Acid (BHBA) levels were measured immediately from the samples taken. BHBA was detected and recorded with an electronic ketone meter (Precision Xceed, Abbott Diabetes Care Ltd., Withney, UK) [23]. Mindray BS-200 (Shenzhen Mindray Animal Medical Technology, China) biochemistry analyzer was used for glucose, alkaline phosphatase (ALP), total bilirubin, direct bilirubin, indirect bilirubin, magnesium, phosphorus, total protein, creatinine, and aspartate transaminase (AST) analyses.

Hormone analysis

The serum samples obtained and stored in labeled microtubes at -80°C (Haier, DW-86L828S, China) were kept at room temperature, thawed, and then vortexed (Hettich Zentrifugen Tuttlingen, D-78532 Eba 20, Germany). Analyzes of LP, RETN, Insulin and AP from serum samples are as follows; Sheep (LP) Elisa Kit (Catalog no: 201-07-006), Sheep (RETN) Elisa Kit (Catalog no: 201-07-0053), Sheep (Insulin) Elisa Kit (Catalog no: 201-07-002) and Sheep (AP) Elisa Kit (Catalog no: 201-07-368) using commercial test kits through sandwich ELISA method by the manufacturer's test procedure (Sunredbio, CHINA). The results were read at 450 nm on the Thermo Scientific Multiskan FC ELISA device. The standard curve range and sensitivity of the tests were 0.1–30 mU·L^{-1} and 0.0866 mU·L^{-1} for Insulin; 2–600 ng·mL^{-1} and 1.857 ng·mL^{-1} for AP; 0.25–70.0 ng·mL^{-1} and 0.244 ng·mL^{-1} for RETN, and 0.05–10 ng·mL^{-1} and 0.05 ng·mL^{-1} for LP, respectively.

Biopsy acquisition and analysis

Liver biopsy samples were taken twice from the animals in the control and PT groups, on the 120th and 143rd d. Within a few minutes after the animals were restrained, liver biopsies were performed with an 18 G (1.2 mm), disposable soft tissue biopsy needle inserted vertically, 9 cm below the processus spinosus, in the tenth intercostal space on the right side of the sheep, as described in Ferreira *et al.* [24]. (Medax, Italy) accompanied by ultrasound (2 Mhz Convex Probe, Mindray DC-N3 Vet, China).

Biopsy samples taken from the liver tissues of sheep were fixed in 10% neutral formalin solution for 24–48 h. Then, it was washed under running water for 8 h to remove the formalin present in the tissue. Tissues were subjected to routine alcohol-xylene follow-up processes and placed in paraffin blocks. After being subjected to deparaffinization in the oven, 5 µm sections taken on slides were stained with hematoxylin-eosin. The histopathological changes seen in the tissues were evaluated under a light microscope (Zeiss Primo Star with an integrated Carl Zeiss Axiocam ERc 5s; Carl Zeiss AG, Oberkochen, Germany) by randomly selecting five different areas as no changes (0), mild (1), moderate (2) and severe (3).

Statistical analysis

The number of animals used in the study was determined using G*Power 3.1 software, with an effect size of 0.3, α<0.05 and 1 – β=0.90, and 8 repeated measurements in 2 groups. The data obtained in the study were used in SPSS 25.0. version (IBM, Armonk, NY, USA) statistical package program and Graphpad Prism version 9.3.1 (Graphpad Software, Inc., CA, USA) [19]. For comparisons of means, repeated analysis of variance with one of the factors in factorial order was used. The suitability of the data for repeated measures analysis of variance was checked using Mauchly's Test of Sphericity. If it did not meet the prerequisites of parametric tests, the degrees of freedom were corrected, and Greenhouse and Geisser tests were applied. Bonferroni correction was made for multiple comparisons. The variables were first checked whether they met the assumptions of normality and homogeneity using the Shapiro wilk and Levene tests. Student's t test was used to evaluate age, body condition score and live weight data. In histopathological measurements, the difference between times was determined by the Kruskal Wallis test, and the time causing the difference was determined by the Mann Whitney U test. Pearson correlation analysis was performed for parametric data to determine the relationship between the parameters. The cut off scoreswere evaluated by receiver operating characteristic (ROC) analysis. Area under the curve value, sensitivity, and specificity values were calculated. Cutting points were evaluated according to the Youden Index. Values of P<0.05 and P<0.01 were accepted for the significance level of the tests.

RESULTS AND DISCUSSION

The mean body weight was 58 ± 4,5 kg (BCS –body condition score–) 3 ± 0.25) in the PT group and 59 ± 3,9 kg (BCS 3 ± 0.25) in the control group, at the beginning of the study. The mean age of the animals in the PT and control groups was 3 ± 1 old year. No statistical difference was observed in body weight, BCS, or age between the two groups at the beginning of the study (P<0.05).

On the 130th d of the study, one animal in the control group was removed from the control group due to widespread infection symptoms such as high fever, leukocytosis, and cessation of eating and drinking water. Two animals in the study group were excluded from the study because they would not be suitable for repeated measurement analysis due to abortion on the 136th and 138th d, respectively. In the study group, on the second day of fasting (d 142) in six animals, standing up with help, licking the wall, leaning their heads against the wall and eating each other's wool were recorded as clinical symptoms. On the third day of starvation (d 143), 4 animals aborted, the study was terminated, and the animals were treated. During the study, no clinical signs of PT were observed in the animals in the control group.

In the histopathological examination of liver tissues, the liver had a normal histological appearance on the 120th and 143rd d of the control group and the 120th d of the PT group, while the nucleus was seen to be pushed towards the cell wall due to severe lipidosis in the hepatocytes on the 143rd d of the PT group (FIG.1, TABLE II).

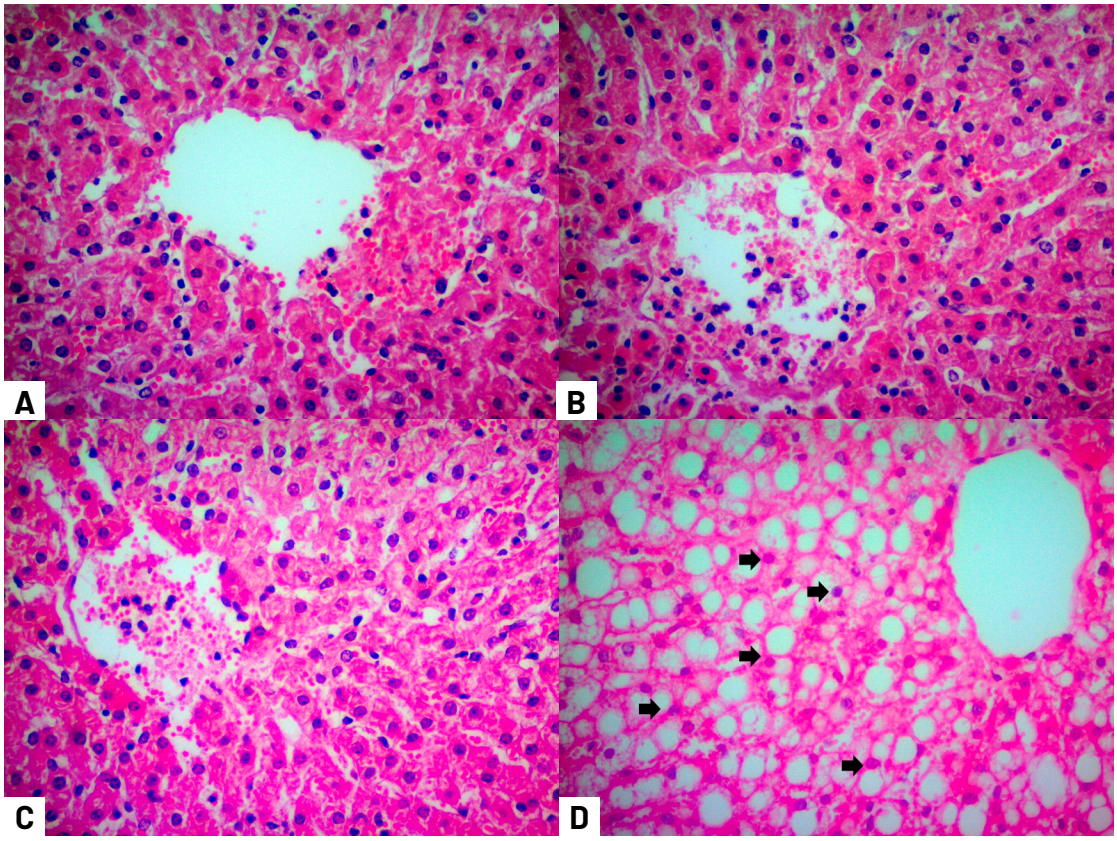

FIGURE 1. A – Control group, 120th day of pregnancy, normal histological appearance. B – Control group, 143rd day of pregnancy, normal histological appearance. C – Pregnancy toxemia (PT) group, 120th day of pregnancy, normal histological appearance. D – PT group, 143rd day of pregnancy, fatty vacuoles in the cytoplasm of hepatocytes and nuclei pushed towards the cell wall (Arrows). Liver – H&E. 40×

TABLE II
Results of liver histopathological examination of ewes in the pregnancy toxemia (PT) group (n=6), which started with restricted feeding on the 120th day of pregnancy and starved between days 140 and 143, and ewes in the control group (n=7), which were adequately fed throughout pregnancy

Days	Fatty Liver Degree
Control group 120th day	0.16±0.40[a]
Control group 143rd day	0.25±0.46[a]
PT group 120th day	0.33±0.51[a]
PT group 143rd day	2.83±0.40[b]

[a,b]: Indicates the difference between times (P<0.05)

Since PT is a metabolic disease that occurs as a result of disorders in carbohydrate and fat metabolism due to negative energy balance and progresses with hypoglycemia and hyperketonemia, liver biopsy is considered the gold standard in detecting fatty liver [28]. Previous studies have reported that starvation or restricted feeding causes the cytoplasm of hepatocytes to fill with fat and their nuclei to be pushed towards the cell wall [28, 29, 30]. In this study, similar histopathological findings were detected in the animals in the PT group. A non-statistically significant observation of fatty liver in the control group led to the conclusion that this condition was associated with the progress of pregnancy.

Correlation analysis was performed to determine the relationship between the parameters in the data set. According to this analysis, a strong negative correlation was found BHBA with glucose (r = -0.94), magnesium (r = -0.89), and ALP (r = -0.91). A strong positive correlation was observed between BHBA with creatinine (r = 0.95), indirect bilirubin (r = 0.96), and total bilirubin (r = 0.95). There was a weak correlation between BHBA with AP and RETN (r = 0.25 and r = 0.16), respectively. A moderate correlation between BHBA and LP with rate of r = 0.61 were determined. A weak correlation was observed between glucose and AP with a rate of r = -0.13, a moderate correlation with leptin with a rate of r = -0.53, and RETN with a rate of r = 0.42. The results of all correlations obtained in the study are shown in Fig. 2 and in TABLE III.

Pregnancy toxemia is known as a serious metabolic condition that has a impact on several physiological systems. PT's overall effects on several physiological systems are reflected in changes to the enzymes of the liver and bile, mineral metabolism, glucose homeostasis, and renal function, as well as in the connections between these biochemical indicators [1, 6, 7]. According to the literature, this study's correlation analysis uncovered a number of significant correlations that highlight the disease's systemic character. These connection outputs support the theory that pregnancy toxemia is a complex metabolic illness.

Serum BHBA, AP, LP, insulin, RETN, glucose, ALP, total bilirubin, magnesium, direct bilirubin, indirect bilirubin, creatinine, phosphorus, AST, and total protein concentration and statical analysis results are given in FIGS. 3, 4, 5, 6.

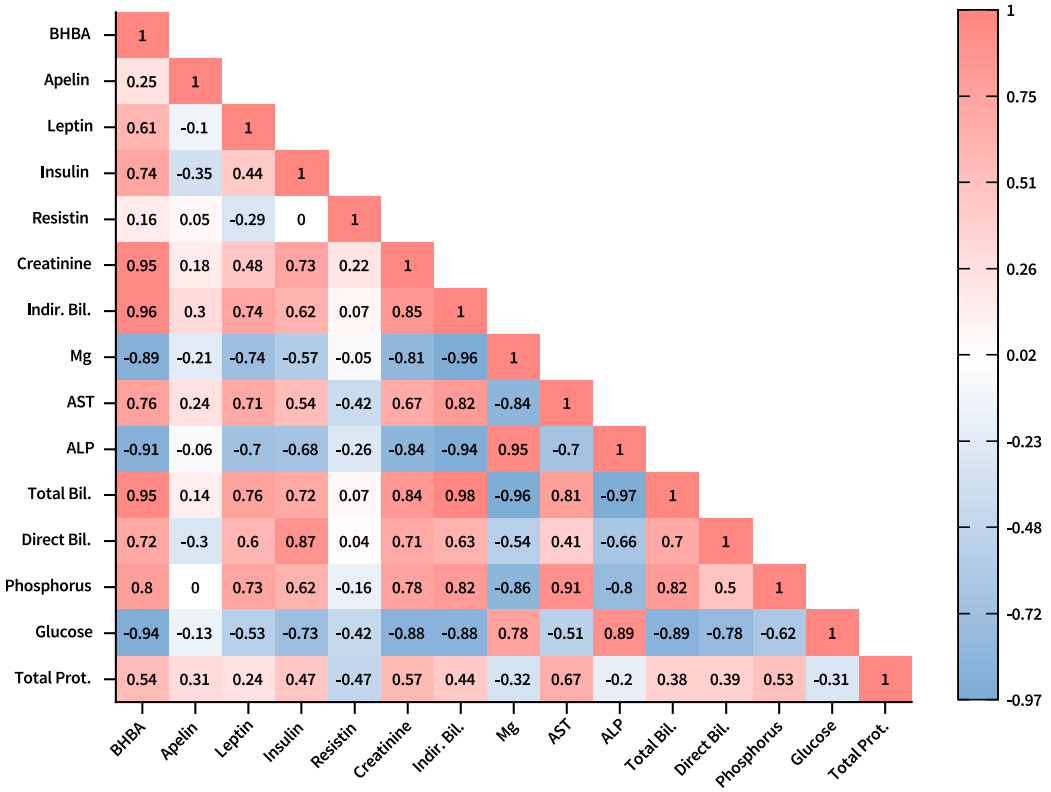

FIGURE 2. Correlations of the data obtained from the ewes in the pregnancy toxemia (PT) group (n=6), which started with restricted feeding on the 120th day of pregnancy and starved between the 140th and 143rd day, and the ewes in the control group (n=7), which were adequately fed throughout pregnancy, and the heat map representation of r values. BHBA: beta hydroxy butyric acid, Indir. Bil.: indirect bilirubin, AST: aspartate transaminase, ALP: alkaline phosphatase, Total Bil.: total bilirubin, Direct Bil.: direct bilirubin and Total Prot.: total protein

TABLE III

***P*-values of the data obtained from the ewes in the pregnancy toxemia (PT) group (n=6), which started with restricted feeding on the 120th day of pregnancy and starved between the 140th and 143rd days, and the ewes in the control group (n=7), which were adequately fed throughout pregnancy**

	BHBA	Apelin	Leptin	Insulin	Resistin	CRE	IND.BIL.	Mg	AST	ALP	TOT.BIL	DIR.BIL	Phos	Glu	TP
BHBA mmol·L^{-1}															
Apelin (ng·L^{-1})	0.552														
Leptin (ng·mL^{-1})	0.110	0.818													
Insulin (U·L^{-1})	0.036	0.390	0.274												
Resistin (ng·mL^{-1})	0.698	0.908	0.481	0.993											
CRE (mg·dL^{-1})	0.000	0.678	0.233	0.040	0.595										
IND.BIL. (mg·dL^{-1})	0.000	0.472	0.035	0.100	0.860	0.007									
Mg (mg·dL^{-1})	0.003	0.612	0.037	0.138	0.915	0.015	0.000								
AST (U·L^{-1})	0.027	0.565	0.048	0.167	0.298	0.068	0.012	0.009							
ALP (U·L^{-1})	0.002	0.888	0.052	0.065	0.536	0.009	0.001	0.000	0.055						
TOT.BIL (mg·dL^{-1})	0.000	0.733	0.029	0.044	0.874	0.010	0.000	0.000	0.016	0.000					
DIR.BIL (mg·dL^{-1})	0.044	0.475	0.117	0.005	0.926	0.046	0.093	0.169	0.310	0.076	0.052				
Phos (mg·dL^{-1})	0.017	0.996	0.039	0.098	0.706	0.024	0.012	0.006	0.002	0.017	0.012	0.212			
Glu (mg·dL^{-1})	0.001	0.763	0.172	0.040	0.296	0.004	0.004	0.023	0.194	0.003	0.003	0.022	0.099		
TP (g·dL^{-1})	0.168	0.451	0.572	0.243	0.238	0.137	0.281	0.440	0.069	0.636	0.357	0.340	0.176	0.460	

Creatinine (CRE), indirect bilirubin (IND.BIL.), magnesium (Mg), aspartate transaminase (AST), alkaline phosphatase (ALP), total bilirubin (TOT.BIL), direct bilirubin (DIR.BIL), phosphorus (Phos), glucose (Glu) and total protein (TP)

When the PT group's restricted feeding began, their BHBA concentration rose, and there was a significant difference (P<0.05) between them and the control group. The BHBA concentration of the control group did not change significantly during the study (P>0.05).

The BHBA concentration started to increase after 5 d of restricted feeding and soon exceeded 1 mmol·L^{-1} in PT group. It reached the critical concentration of 1.6 mmol·L^{-1} at the end of 10 d and rose above 3.2 mmol·L^{-1} on the 143rd d when the study was terminated. (FIG. 4).

Based on the 0.8 mmol·L^{-1} BHBA threshold reported in the literature Duehlmeier *et al*. [25], ROC analyzes of the variables in the data set were performed and sensitivity, specificity, cut off, positive likelihood ratio and negative likelihood ratio (+, –) were calculated. ROC analysis results presented in TABLE IV. and FIGS. 7, 8.

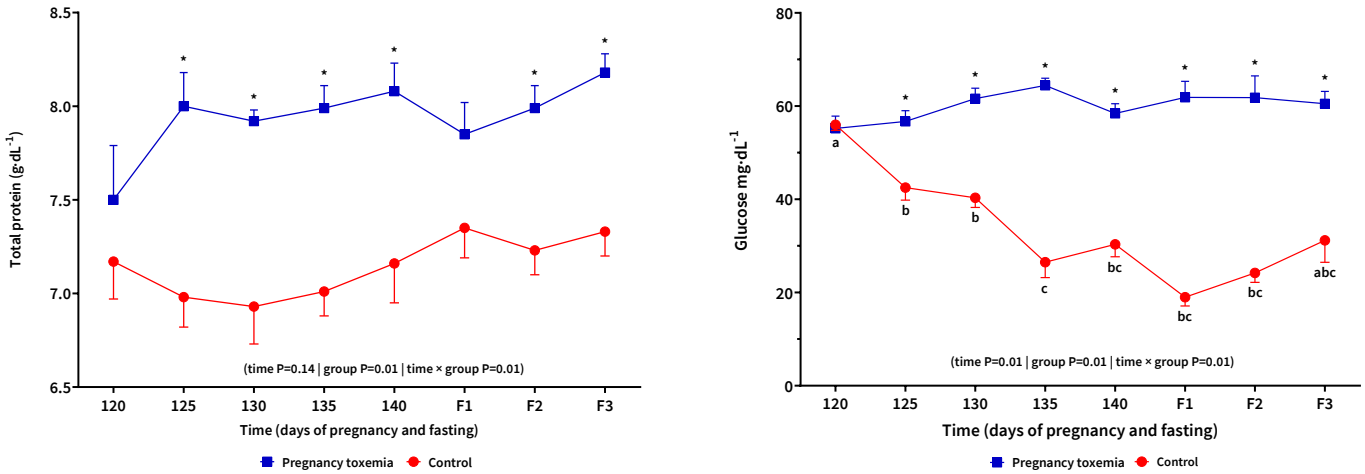

FIGURE 3. Changes in total protein and glucose concentrations in the study group twin pregnant ewes, which were subjected to restricted feeding from 120th.days of pregnancy to 140th days and starved after 140th days of pregnancy, and in the control group twin pregnant ewes, which were fed with healthy food during the same period. Error bars show SEM, PT: Pregnancy toxemia F: Fasting, *: *P*<0.05, abc: Varied characters under the same line are statically different. (Time = Effect of time independent of groups, Group = Effect of group independent of time, Time × Group = Interaction between groups over time)

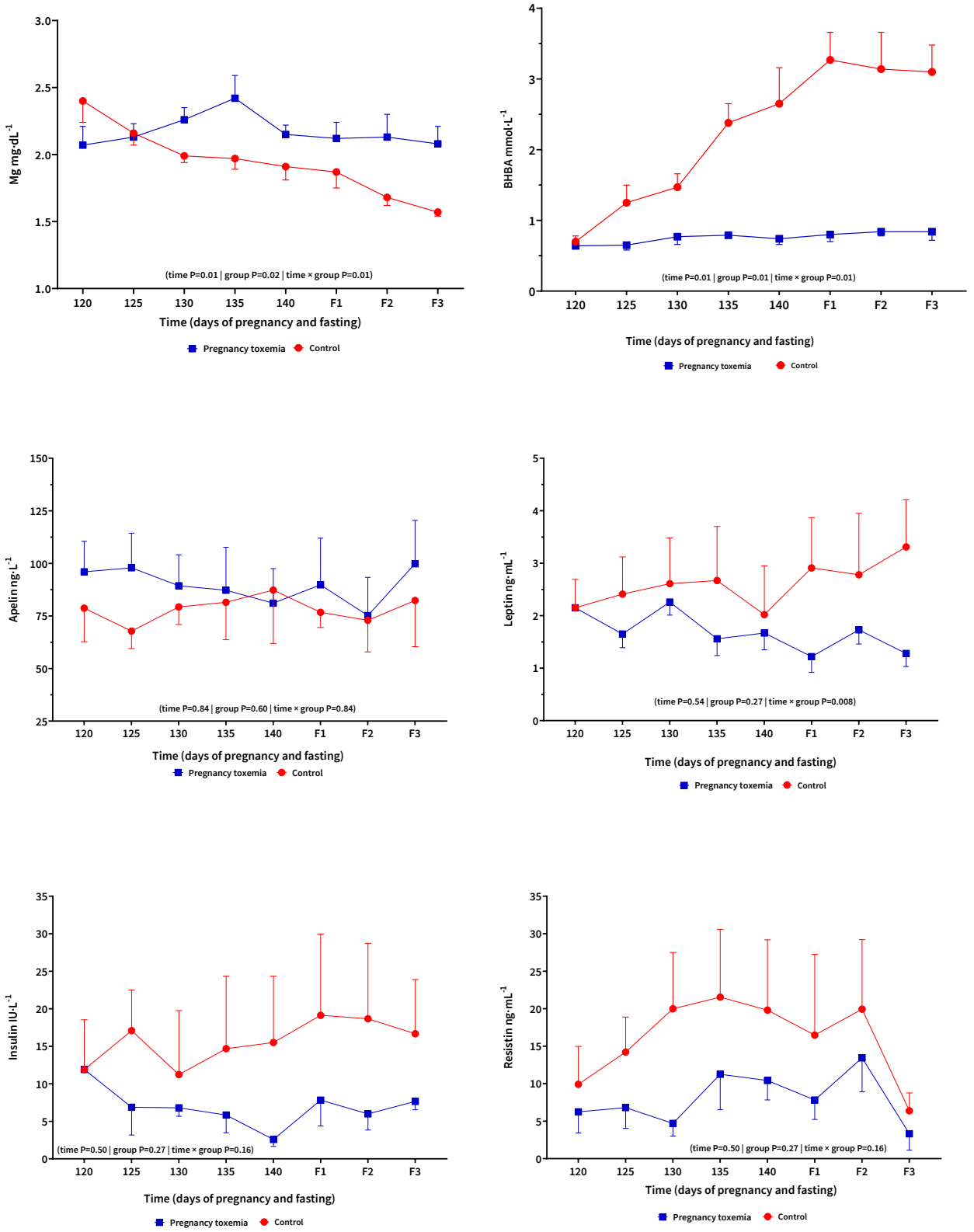

FIGURE 4. Changes in magnesium (Mg), beta hydroxy butyric acid (BHBA), apelin, leptin, resistin, insulin concentrations in the study group twin pregnant ewes, which were subjected to restricted feeding from 120th.days of pregnancy to 140th days and starved after 140th days of pregnancy, and in the control group twin pregnant ewes, which were fed with healthy food during the same period. Error bars show SEM, PT: Pregnancy toxemia F: Fasting, *: *P*<0.05, abc: Varied characters under the same line are statically different. (Time = Effect of time independent of groups, Group = Effect of group independent of time, Time × Group = Interaction between groups over time)

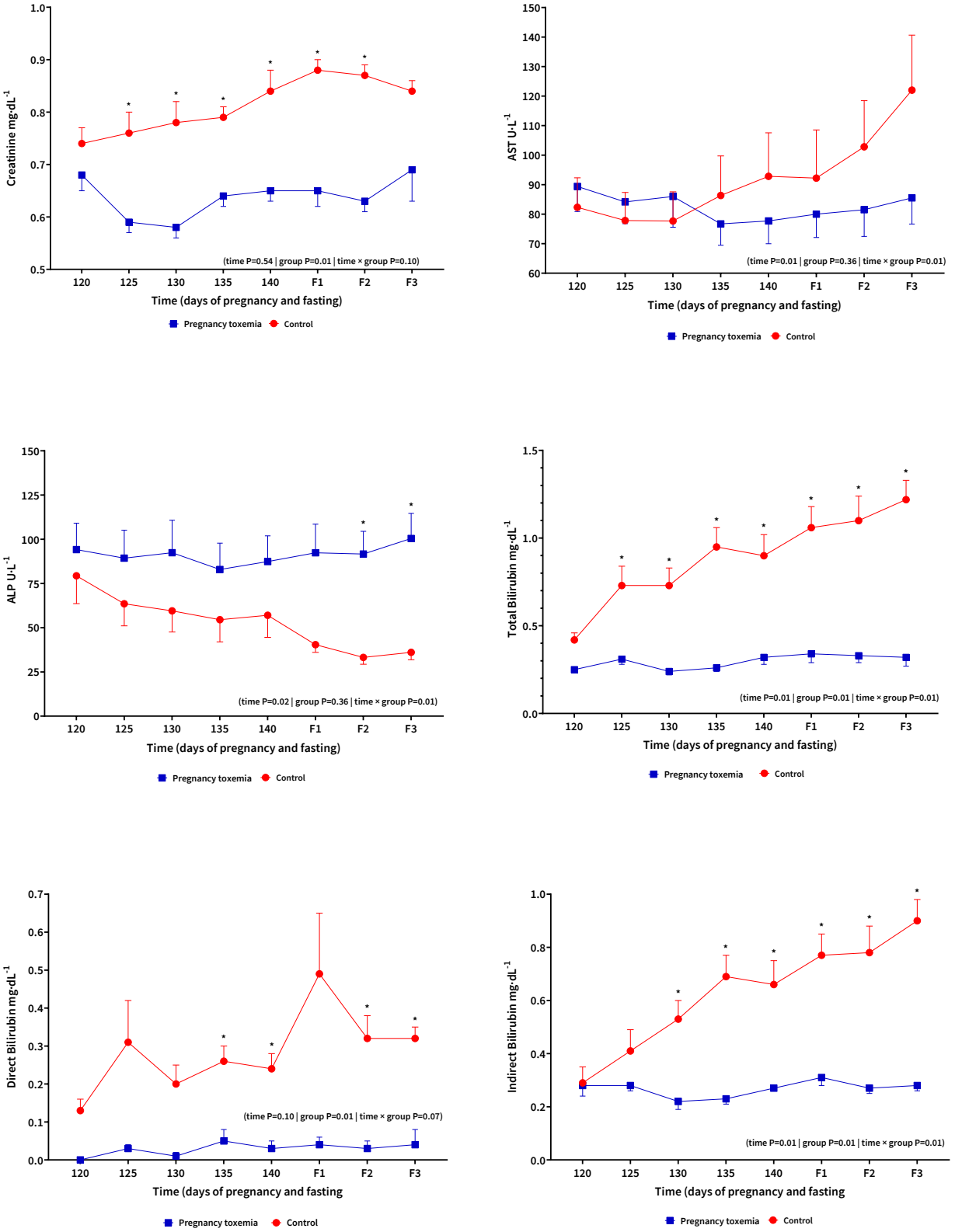

FIGURE 5. Changes in creatinine, aspartate transaminase (AST), alkaline phosphatase (ALP), total bilirubin, direct bilirubin and indirect bilirubin concentrations in the study group twin pregnant ewes, which were subjected to restricted feeding from 120 d of pregnancy to 140 d and starved after 140 d of pregnancy, and in the control group twin pregnant ewes, which were fed with healthy food during the same period. Error bars show SEM. PT: Pregnancy toxemia F: Fasting *: *P*<0.05(Time = Effect of time independent of groups, Group = Effect of group independent of time, Time x Group = Interaction between groups over time)

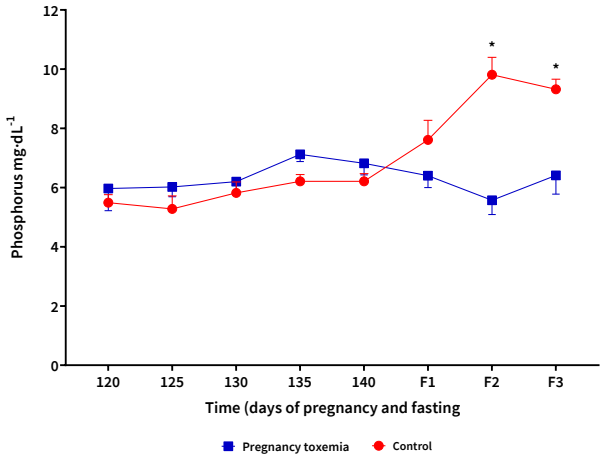

FIGURE 6. Changes in phosphorus concentrations in the study group twin pregnant ewes, which were subjected to restricted feeding from 120 d of pregnancy to 140 d and starved after 140 d of pregnancy, and in the control group twin pregnant ewes, which were fed with healthy food during the same period. Error bars show SEM. PT: Pregnancy toxemia F: Fasting *: *P*<0.05(Time = Effect of time independent of groups, Group = Effect of group independent of time, Time × Group = Interaction between groups over time)

As a result of the study, BHBA levels increased significantly in sheep in the PT group with restricted feeding and starvation. In liver biopsy analysis, steatosis was evident in sheep in the PT group. Glucose, magnesium, total protein and ALP levels of biochemical parameters were found to be significantly lower than the control group. A significant increase was detected in total bilirubin, direct bilirubin, indirect bilirubin, AST and phosphorus levels in the PT group. Although creatinine levels increased over time, no difference was detected between groups. There was no difference in AP, RETN and Insulin levels between the groups in the measurements. At the LP level, there was a statistically significant difference between the groups, especially after the fasting application.

The frequently preferred method to establish experimental PT is to fast for 72 h on the 140th d of pregnancy [26, 27]. In previous study with study team Kivrak *et al.* [19], 20 d of restricted feeding and 96 hours of fasting were applied to singleton pregnant sheep to create PT. This approach was chosen because, in field settings, there is no abrupt hunger; instead, the animals' negative energy balance builds up until they finally starve. Since this study was conducted on twin pregnant sheep, the fasting period was planned as 72 h, unlike previous study, anticipating that the symptoms would occur more quickly.

β–Hydroxy Butyric Acid levels resulting from the mobilization of fats are considered a good indicator in showing NEB (Negative energy balance) [1]. On the first day of the study (120th d of pregnancy), the BHBA level was below 0.8 mmol·L^{-1} in both groups. Although there was no significant change in the BHBA level as pregnancy progressed in the control group, the BHBA level exceeded 3 mmol·L^{-1} on the 141st d in the PT group. The development of clinical symptoms, the occurrence of abortion, and the buildup of fat in the liver were assessed as proof that the technique employed in this study was appropriate for inducing PT in twin pregnant sheep.

Previous studies have reported that BHBA levels and clinical findings are not compatible. In some studies, it has been reported that clinical symptoms begin to appear between BHBA levels of 0.8 mmol·L^{-1} and 1.6 mmol·L^{-1} [31], and in others, they are observed only after the level of 3 mmol·L^{-1} is exceeded [32]. In the current study, abortion was observed in two sheep when BHBA was between 0.8–1.6 mmol·L^{-1}

TABLE IV
Optimum cut off values and respective sensitivity, specificity, area under the curve, SE, positive likelihood ratio and negative likelihood ratio of the Pregnancy toxemia (PT) (beta hyrdoxy butyric acid (BHBA) threshold is 0.8 mmol/l) prediction in last trimester pregnancy in Akkaraman–Kangal ewes

Parameters	Cutoff	AUC	SE	%95 CI for AUC	P	Sensitivity% (%95 CI)	Spesificity% (%95 CI)	LR+	LR–
Resistin (ng·mL^{-1})	8.740	0.555	0.0672	0.423 – 0.686	=0.386	55.56	60.00	1.38	0.74
Insulin (U·L^{-1})	5.971	0.589	0.0657	0.460 – 0.718	=0,158	52.78	60.00	1.31	0.78
Apelin (ng·L^{-1})	87.60	0.529	0.0657	0.400 – 0.658	=0.642	52.78	62.00	1.38	0.76
Leptin (ng·mL^{-1})	1.386	0.644	0.0607	0.525 – 0.763	=0.023*	69.44	56.00	1.57	0.63
IND.BIL. (mg·dL^{-1})	0.465	0.929	0.0322	0.866 – 0.992	<0.0001**	81.58	97.56	33.45	0.19
CRE (mg·dL^{-1})	0.735	0.915	0.0306	0.855 – 0.975	<0.0001**	81,58	87.23	6.39	0.21
TOT.BIL (mg·dL^{-1})	0.420	0.975	0.0130	0.950 – 1.000	<0.0001**	97.37	82.98	5.72	0.03
DIR.BIL (mg·dL^{-1})	0.165	0.957	0.0199	0.918 – 0.996	<0.0001**	89.19	89.13	8.20	0.12
TP (g·dL^{-1})	7.435	0.874	0.0380	0.799 – 0.948	=0.0001**	84.21	82.98	4.94	0.19
Glu (mg·dL^{-1})	48.50	0.988	0.0087	0.971 – 1.000	<0.0001**	94.74	95.74	22.26	0.05
Mg (mg·dL^{-1})	2.020	0.762	0.0523	0.659 – 0.864	<0.0001*	71.05	63.83	1.96	0.45
Phos (mg·dL^{-1})	6.495	0.577	0.0639	0.452 – 0.703	=0.219	52.63	53.19	1.12	0.89
ALP (U·L^{-1})	56.75	0.815	0.0482	0.721 – 0.910	<0.0001**	78.95	74.47	3.09	0.28
AST (U·L^{-1})	83.00	0.636	0.0626	0.513 – 0.759	=0.031*	57.89	57.45	1.36	0.73

AUC: area under the curve, LR+: positive likelihood ratio, LR-: negative likelihood ratio, SE: standard error, CI: confidence interval, *P*: significance, indirect bilirubin (IND.BIL.), Creatinine (CRE), magnesium (Mg), aspartate transaminase (AST), alkaline phosphatase (ALP), total bilirubin (TOT.BIL), direct bilirubin (DIR.BIL), phosphorus (Phos), glucose (Glu) and total protein (TP)

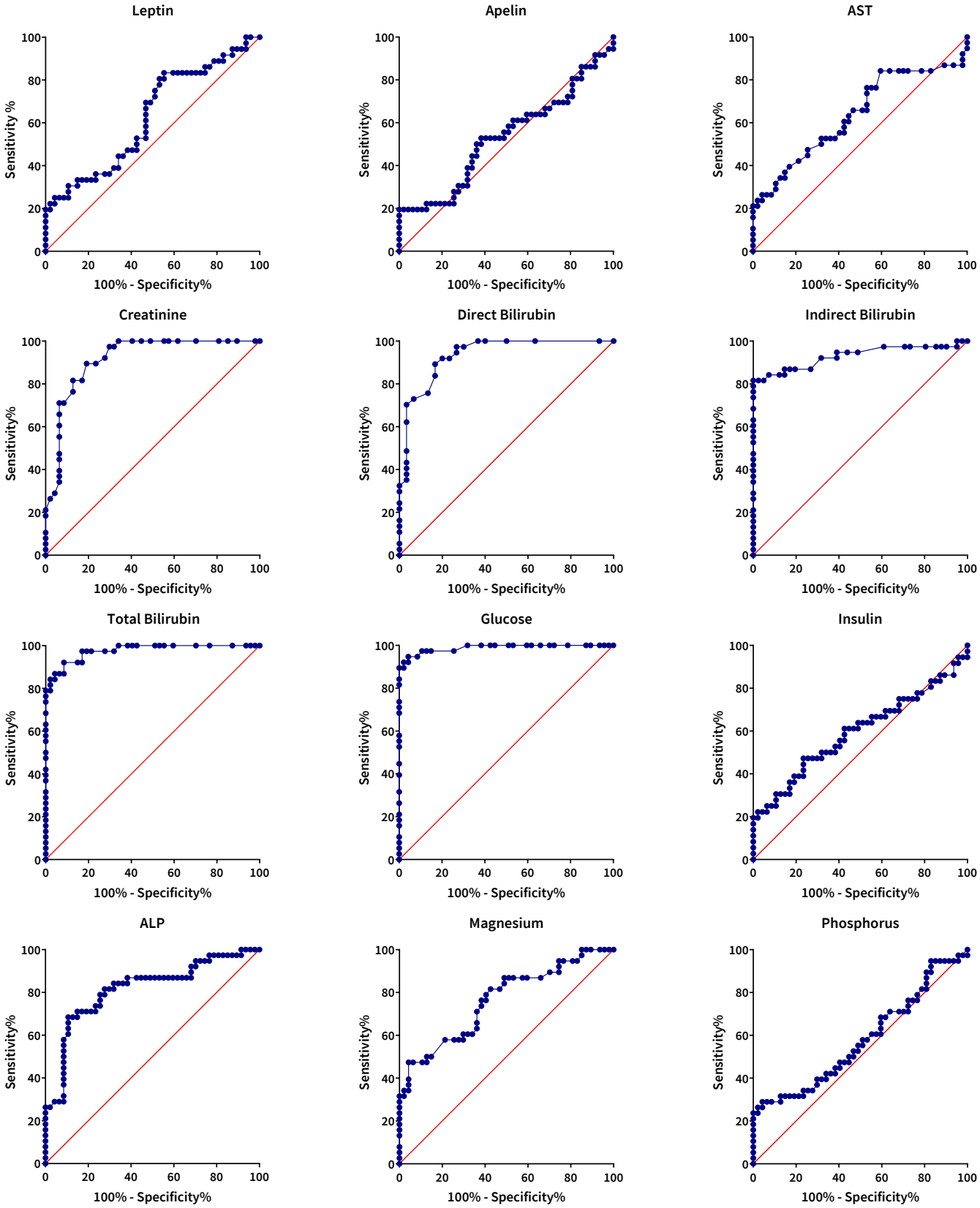

FIGURE 7. ROC analysis of leptin, apelin, aspartate transaminase (AST), creatinine, direct bilirubin, indirect bilirubin, total bilirubin, glucose, insulin, alkaline phosphatase (ALP), magnesium, phosphorus in Akkaraman–Kangal breed sheep in the last trimester of pregnancy, based on the 0.8 mmol threshold

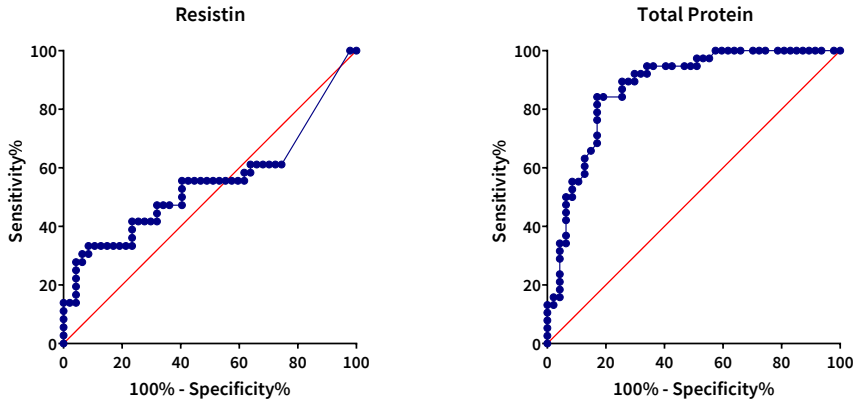

FIGURE 8. ROC analysis of resistin and total protein in Akkaraman–Kangal breed sheep in the last trimester of pregnancy, based on the 0.8 mmol threshold.

(BHBA: 1.4 mmol·L^{-1} and 1.5 mmol·L^{-1}). In other sheep, clinical symptoms began to appear only when the BHBA level increased to 3 mmol·L^{-1} and above. Therefore, in this study, in line with Öztürk and Mamak [31] and Sargison [33], it was confirmed that even when the BHBA level is between 0.8–1.6 mmol·L^{-1}, it indicates an energy deficiency, and clinical signs (such as abortion) can be observed in animals.

Glucose level is one of the frequently measured parameters in PT. Researchers have found different results regarding glucose levels in PT studies. Some researchers reported hypoglycemia, some reported normoglycemia, and some reported hyperglycemia [1, 34, 35]. Additionally, it has been reported that it is not compatible with BHBA due to the variability of glucose level [36]. In the current study, it was observed that glucose levels gradually decreased with the onset of restricted feeding; It was observed that when fasting started, it started to increase, but this increase did not reach hyperglycemia or even normoglycemia. On the other hand, this study revealed that glucose showed a very strong negative correlation with BHBA level. The following explanation can be used to describe the glucose testing situation: In animals, hypoglycemia first appears when NEB starts. Long-term lipomobilization and hypoglycemia lead to insulin resistance. Tissues that are resistant to insulin are less sensitive to it. Insulin causes a decrease in glucose transport to tissues and a rise in blood glucose levels. For this reason, studies have reported that normoglycemia or hyperglycemia may occur [1].

Adult sheep have a limited ability to circulate body magnesium reserves. Therefore, they need to take magnesium daily to meet their requirements [37]. Decreased magnesium concentration may cause fatigue, difficulty standing up, lying on one side, tetany, and seizures [38]. Additionally, hypomagnesemia impairs both insulin secretion and the effect of insulin on peripheral tissues, thus affecting the development of poor insulin resistance. In this study, the magnesium level decreased gradually with 20 d of restricted feeding and became evident with 3 d of complete starvation. Additionally, in this study, a strong negative correlation was observed with BHBA level. On the other hand, magnesium levels were reported as normal in studies conducted on sheep with subclinical PT [36, 39]. However, in clinical PT cases [40], it has been reported that magnesium levels may decrease due to decreased food intake in the last period of pregnancy [38]. Therefore, with the current study, it was concluded that the severity of PT and magnesium concentration were compatible.

Since the organ most affected by gestational toxemia is the liver, tests showing the status of the liver have been shown in many studies [28, 35, 41, 42]. In the current study, total protein, total bilirubin, indirect bilirubin, direct bilirubin, AST and ALP levels were measured and ROC analysis was performed for their diagnostic significance. In previous studies, it was stated that total protein decreases because the decrease in hepatic metabolism and impairment of its function causes a decrease in protein synthesis [41, 42]. As a matter of fact, similar results were obtained in this study. In the current study, it was determined that total bilirubin, indirect bilirubin and direct bilirubin levels were high. Constable *et al.* [43], suggested that the bilirubinemia that occurs is caused by the cessation of bile flow as a result of the expansion of hepatocytes infiltrated with large amounts of fat. AST enzyme is considered the most sensitive enzyme to histopathological changes in the liver. Studies on the subject have shown that AST increases due to fat accumulation in hepatocytes, accompanied by mitochondrial dysfunction and destruction of cellular organelles [41, 44]. In this study, the data obtained regarding AST were found to be compatible with the literature findings. On the other hand, there are conflicting results in studies on serum ALP levels. In the current study, ALP level was found to be significantly lower in the PT group compared to the control group. Sargison *et al.* [45], stated that high ALP levels may be an indicator of poor prognosis for the mother. There are studies also reporting that low ALP levels indicate fetal growth retardation or a high-risk pregnancy [46, 47]. In other words, it can be said that high ALP levels are an indicator of a bad prognosis for the mother and low ALP levels are an indicator of a bad prognosis for the fetus. Considering the ALP findings in this study, the pattern of abortion in the PT group was found to be compatible with the literature information in question. In addition, in this study, it was concluded that BHBA and liver panel tests were highly correlated and were effective auxiliary parameters in reaching the diagnosis, in line with the literature.

Researchers have reported conflicting results on creatinine and phosphorus concentrations in previous studies [27, 32, 39, 40, 48]. In the current study, although serum creatinine levels differed on the d when they were measured (130th, 135th, 140th, 141st and 142nd), there was no statistical difference between the groups. It is reported in the literature that the increase in creatinine level is a result of protein catabolism and renal failure [40]. When nephron loss reaches 75%, creatinine levels rise. In cases of renal failure with less nephron

loss, the creatinine level may be normal [48]. Contrary to studies in which high creatinine levels were associated with death [36, 41], creatinine levels remained normal between groups in this study. In the current study, it was observed that the phosphorus level increased statistically significantly with fasting. It has been reported that phosphorus level increases due to hyperketonemia, decreased calcium absorption, increased parathormone activity and decreased glomerular filtration [35, 49, 50]. It is known that hypocalcemia and hyperketonemia are common in PT. In the current study, it was concluded that the formation of hyperphosphatemia, especially during the fasting period, is an indicator of impaired phosphorus balance caused by hyperketonemia.

Previous studies reported that insulin levels decreased in sheep affected by PT [35]. On the other hand, in a study conducted on pregnant sheep where 50% of their energy requirements were met, it was reported that the insulin level was not affected [51]. As a matter of fact, in this study, there was no difference between groups in insulin concentration. In the light of these studies, it has been evaluated that hypoinsulinemia is more prominent in severe PT cases accompanied by hyperglycemia. Hyperglycemia did not occur in animals with PT during the current study. Therefore, it suggests that the insulin level remains at basal level.

In previous studies, there are different results regarding the RETN level in situations of restricted feeding or starvation. In a study using fat-tailed sheep, it was found that the RETN level increased 5.2 times compared to the control group after 4 weeks of fasting [52]. On the contrary, a decrease in RETN level has been reported in rats fasted for a long time [53]. In another study, RETN levels were shown to be stable during fasting [54]. In the current study, no difference was seen between the groups in the RETN level. It has been reported that high levels of insulin, the regulator of serum RETN expression, may increase RETN concentration [55]. In this study, it was concluded that there was no change in RETN level as there was no change in insulin level.

Apelin, secreted from adipocytes, is found in the heart, lung, kidney, liver, adipose tissue, digestive system, brain, adrenal glands, endothelium and plasma and plays an important role in the physiology of the control of feeding behavior, energy expenditure and regulation of body fluid homeostasis [56]. In previous studies, it was reported that high levels of insulin, which is the regulator of serum AP expression, decreases AP concentration, while low insulin levels increase AP levels [57]. In the current study, no change in AP level was observed between groups. In this study, it was concluded that there was no change in AP level between the groups in parallel with the insulin level.

Previous studies have reported that plasma LP level decreases due to increased energy need [58, 59]. Consistent with this information, in the current study, a gradual decrease in LP level was observed in the control group as birth approached. In the control group, due to the increase in energy needs, the BHBA level was measured as 0.64 mmol·L^{-1} on the 120th d of pregnancy, while the average was 0.76 mmol·L^{-1} on the 143rd d of pregnancy. In the PT group, although the LP level was similar to the control group during the restricted feeding period, it was determined that it decreased statistically significantly during the fasting period. In previous studies, it was reported that the LP level decreased due to negative energy balance [60, 61]. On the other hand, another study reported that there was no change in the serum LP level of sheep after 5 days of fasting [62]. In a different study on LP level, the plasma LP level of sheep in the anoestrus period and the mating season was compared and it was reported that it was 180% higher in the anoestrus period [63]. High LP levels were expressed as LP resistance and were reported to be a result of seasonal adaptation, which provides sheep with more nutrition opportunities and contributes to energy storage [64]. Prolonged hypoglycemia and correspondingly increased cortisol levels in pregnancy toxemia are well known [65, 66]. Cortisol hormone and leptin levels show an inverse correlation in acute and subacute cases. However, long-term exposure to cortisol increases leptin synthesis [67, 68]. Although the process was short in previous studies, in the present study it was concluded that it covered a 23 d period and was carried out during the anoestrus period, contributing to the increase in LP level.

CONCLUSION

β-Hydroxy Butyric Acid and glucose are simple and effective biomarkers of maternal energy deprivation. This study revealed that while glucose levels decreased and BHBA levels increased with restricted feeding, there was no change in AP, RETN and Insulin concentrations. This study showed that monitoring LP, liver panel tests, kidney function tests, and minerals in addition to BHBA and glucose in PT may aid diagnosis. Low ALP concentration may be a sign that the health of the fetus is in danger in sheep. More experimental animals and more detailed studies are needed to fully understand the role of adipokines, especially LP, in pregnancy toxaemia of sheep.

ACKNOWLEDGMENTS

The authors thank to Erciyes University Betül Ziya Eren Genome and Stem Cell Research Center for assitance and contributions with the laboratory analysis. (GENKÖK), Kayseri, Türkiye.

Funding

This study that produced by first author's Ph.D. thesis named; "Investigation of Serum Adipokine Concentrations in Experimentally Induced Pregnancy Toxemia of Akkaraman – Kangal Ewes." was supported by The Coordination Unit of Scientific Research Projects, Erciyes University (Project No. TDK-2022-12347).

Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethics Approval

The study protocol was approved by the Animal Experiments Local Ethics Committee of Sivas Cumhuriyet University (Approval number 656; Approval date, 17.06.2022).

BIBLIOGRAPHIC REFERENCES

[1] Ji X, Liu N, Wang Y, Ding K, Huang S, Zhang C. Pregnancy Toxemia in Ewes: A Review of Molecular Metabolic Mechanisms and Management Strategies. Metabolites. [Internet]. 2023; 13(2):149. doi: https://doi.org/g5rcpq

[2] Takci A, Kivrak MB, Murat H, Cizmeci SU. Reproductive and economic evaluation of sexual stimulation during the anestrous period in a commercial farm with neonatal lamb losses. Arq. Bras. Med. Vet. Zoot. [Internet]. 2023; 75(4):687-695. doi: https://doi.org/g5rcpr

[3] Takci A, Kivrak MB. Determination of the efficacy of human Chorionic Gonadotropin (hCG) administrations on reproductive performance, placentation, parturition, and neonatal parameters on different post-mating days in Kangal ewes sexually induced during anestrus. Rev. Cient. FCV-LUZ. [Internet]. 2023; 33(2):e33275. doi: https://doi.org/g5rcps

[4] Rook JS. Pregnancy toxemia of ewes, does, and beef cows. Vet. Clin. North. Am. Food Anim. Pract. [Internet]. 2000; 16(2):293–317. doi: https://doi.org/njpx

[5] Schlumbohm C, Harmeyer J. Twin-pregnancy increases susceptibility of ewes to hypoglycaemic stress and pregnancy toxaemia. Res. Vet. Sci. [Internet]. 2008; 84(2):286–299. doi: https://doi.org/czcs8p

[6] Fasshauer M, Blüher M. Adipokines in health and disease. Trends Pharmacol. Sci. [Internet]. 2015; 36(7):461–470. doi: https://doi.org/gh2fcr

[7] Galic S, Oakhill JS, Steinberg GR. Adipose tissue as an endocrine organ. Mol. Cell. Endocrinol. [Internet]. 2010; 316(2):129–139. doi: https://doi.org/bssxr7

[8] Mattu HS, Randeva HS. Role of adipokines in cardiovascular disease. J. Endocrinol. [Internet]. 2013; 216(1):17–36. doi: https://doi.org/f4gsr3

[9] O'Dowd BF, Heiber M, Chan A, Heng HHQ, Tsui LC, Kennedy JL, Shi X, Petronis A, George SR, Nguyen T. A human gene that shows identity with the gene encoding the angiotensin receptor is located on chromosome 11. Gene [Internet]. 1993; 136(1–2):355–360. doi: https://doi.org/dc2sxm

[10] Tekin S, Erden Y, Sandal S, Etem Onalan E, Ozyalin F, Ozen H, Yilmaz B. Effects of apelin on reproductive functions: relationship with feeding behavior and energy metabolism. Arch. Physiol. Biochem. [Internet]. 2017; 123(1):9–15. doi: https://doi.org/g5rcp4

[11] Steppan CM, Bailey ST, Bhat S, Brown EJ, Banerjee RR, Wright CM, Patel HR, Ahima RS, Lazar MA. The hormone resistin links obesity to diabetes. Nature. [Internet]. 2001; 409(6818):307–312. doi: https://doi.org/cctkn4

[12] Mostafazadeh M, Haiaty S, Rastqar A, Keshvari M. Correlation between resistin level and metabolic syndrome component: a review. Horm. Metab. Res. [Internet]. 2018; 50(7):521–536. doi: https://doi.org/gdtwtv

[13] Fujita H, Fujishima H, Morii T, Koshimura J, Narita T, Kakei M, Ito S. Effect of metformin on adipose tissue resistin expression in db/db mice. Biochem. Biophys. Res. Commun. [Internet]. 2002; 298(3):345–349. doi: https://doi.org/fd8q9m

[14] Obradovic M, Sudar-Milovanovic E, Soskic S, Essack M, Arya S, Stewart A, Gojobori T, Isenovic ER. Leptin and obesity: role and clinical implication. Front. Endocrinol. [Internet]. 2021; 12:585887. doi: https://doi.org/gqfdz9

[15] Mongini A, Van Saun RJ. Pregnancy toxemia in sheep and goats. Vet. Clin. N. Am. Food Anim. Pract. [Internet]. 2023; 39(2):275–291. doi: https://doi.org/g5rcp6

[16] Olfati A, Moghaddam G, Bakhtiari M. Diagnosis, treatment and prevention of pregnancy toxemia in ewes. Int. J. Adv. Biol. Biomed. Res. [Internet]. 2013 [cited 24 Feb. 2024]; 1(11):1452–1456. Available in: https://goo.su/ghjCsqt

[17] Ekici M, Takcı A, Kıvrak MB. Comparison of some hematological and serum biochemical variables in Kangal Akkaraman, Texel and Île De France ewes in lactation period within Sivas province. Eurasian J. Vet. Sci. [Internet]. 2021; 37(4):296–302. doi: https://doi.org/g5rcp8

[18] Ferguson JD, Galligan DT, Thomsen N. Principal descriptors of body condition score in Holstein cows. J. Dairy Sci. [Internet]. 1994; 77(9):2695–2703. doi: https://doi.org/dm64q7

[19] Kivrak MB, Turk S, Takci A, Bolukbas B, Agaoglu RT, Coskun A. Pulse wave Doppler ultrasound of umbilical cord in experimentally induced pregnancy toxemia in sheep. Res. Vet. Sci. [Internet]. 2023; 160:18–25. doi: https://doi.org/g5rcqc

[20] National Research Council. Nutrient requirements of small ruminants: sheep, goats, cervids, and new world camelids [Internet]. Washington (DC, USA): Nat. Acad. Press. 2007; 384 p. doi: https://doi.org/gjrvgb

[21] AOAC International. Official methods of analysis of AOAC International. 16th ed. Gaithersburg (MD, USA): AOAC International; 1995. 1015 p.

[22] Mertens DR. Gravimetric determination of amylase-treated neutral detergent fiber in feeds with refluxing in beakers or crucibles: collaborative study. J. AOAC Int. 2002; 85(6):1217–1240. PMID: 12477183

[23] Pichler M, Damberger A, Schwendenwein I, Gasteiner J, Drillich M, Iwersen M. Thresholds of whole-blood β-hydroxybutyrate and glucose concentrations measured with an electronic hand-held device to identify ovine hyperketonemia. J. Dairy Sci. [Internet]. 2014; 97(3):1388–1399. doi: https://doi.org/g5rcqd

[24] Ferreira AV, Van der Merwe HJ, Slippers SC. A technique for obtaining liver biopsies from mature sheep. Small Rumin. Res. [Internet]. 1996; 22(1):89–92. doi: https://doi.org/fwxvzh

[25] Duehlmeier R, Fluegge I, Schwert B, Ganter M. Insulin sensitivity during late gestation in ewes affected by pregnancy toxemia and in ewes with high and low susceptibility to this disorder. J. Vet. Intern. Med. [Internet]. 2013; 27(2):359–366. doi: https://doi.org/f4rb2w

[26] González FHD, Hernández F, Madrid J, Martínez-Subiela S, Tvarijonaviciute A, Cerón JJ, Tecles F. Acute phase proteins in experimentally induced pregnancy toxemia in goats. J. Vet. Diagn. Investig. [Internet]. 2011; 23(1):57–62. doi: https://doi.org/dsbjxx

[27] Hefnawy AE, Youssef S, Shousha S. Some immunohormonal changes in experimentally pregnant toxemic goats. Vet. Med. Int. [Internet]. 2010; 2010:768438. doi: https://doi.org/cj3gcn

[28] Cal L, Borteiro C, Benech A, Rodas E, Abreu MN, Cruz JC, González-Montaña JR. Histological changes of the liver and metabolic correlates in ewes with pregnancy toxemia. Arq. Bras. Med. Vet. Zoot. [Internet]. 2009; 61(2):306–312. doi: https://doi.org/d3cwfs

[29] Kabakci N, Yarim G, Yarim M, Duru O, Yagci B, Kisa U. Pathological, clinical and biochemical investigation of naturally occurring pregnancy toxemia of sheep. Acta Vet. [Internet]. 2003; 53(2–3):161–169. doi: https://doi.org/fdqpp3

[30] Wang M, Li E, Wang G. Histopathological analysis of liver during pregnancy toxemia in small-tailed han sheep. Agric. Sci. Technol. [Internet]. 2014 [cited 15 Mar. 2024]; 15(3):470–473. Available in: https://goo.su/C47SWX

[31] Öztürk M, Mamak N. Current energy and lipid metabolism biomarkers in sheep with subclinical and clinical pregnancy toxemia. Med. Weter. [Internet]. 2023; 79(3):123–129. doi: https://doi.org/g5ggcb

[32] Souto RJC, Afonso JAB, Mendonça CL, Carvalho CCD, Silva-Filho AP, Cajueiro JFP, Lima EHF, Soares PC. Achados bioquímicos, eletrolíticos e hormonais de cabras acometidas com toxemia da prenhez. [Biochemical, electrolytic and hormonal findings in goats affected with pregnancy toxemia]. Pesq. Vet. Bras. [Internet]. 2013 [cited 12 Feb. 2024]; 33(10):1174–1182. Portuguese. Available in: https://goo.su/3HAl7Z

[33] Sargison ND. Pregnancy toxaemia. Aitken ID, editor. Diseases of sheep. 4th ed. Oxford (United Kingdom): Blackwell Publishing; 2007. p. 359–362.

[34] Henze P, Bickhardt K, Fuhrmann H, Sallmann HP. Spontaneous pregnancy toxaemia (ketosis) in sheep and the role of insulin. J. Vet. Med. A [Internet]. 1998; 45(5):255–266. doi: https://doi.org/bnrk88

[35] Souto RJC, Afonso JAB, Mendonça CL, Dantas AFM, Cajueiro JFP, Gonçalves DNA, Olivera Filho EF, Soares PC. Biochemical, endocrine, and histopathological profile of liver and kidneys of sheep with pregnancy toxemia. Pesq. Vet. Bras. [Internet]. 2019; 39(10):780–788. doi: https://doi.org/g5rcqj

[36] Iqbal R, Beigh SA, Mir AQ, Shaheen M, Hussain SA, Nisar M, Dar AA. Evaluation of metabolic and oxidative profile in ovine pregnancy toxemia and to determine their association with diagnosis and prognosis of disease. Trop. Anim. Health Prod. [Internet]. 2022; 54(6):338. doi: https://doi.org/g5ggb8

[37] Friend MA, Bhanugopan MS, McGrath SR, Hocking Edwards J, Hancock S, Loudon K, Miller D, Mcgilchrist P, Refshauge G, Robertson SM, Thompson AN, Masters DG. Do calcium and magnesium deficiencies in reproducing ewes contribute to high lamb mortality? Anim. Prod. Sci. [Internet]. 2020; 60(6):733–751. doi: https://doi.org/g5rcqp

[38] Brozos C, Mavrogianni VS, Fthenakis GC. Treatment and control of peri-parturient metabolic diseases: pregnancy toxemia, hypocalcemia, hypomagnesemia. Vet. Clin. N. Am. Food Anim. Pract. [Internet]. 2011; 27(1):105–113. doi: https://doi.org/d95tfz

[39] Bani Ismail ZA, Al-Majali AM, Amireh F, Al-Rawashdeh OF. Metabolic profiles in goat does in late pregnancy with and without subclinical pregnancy toxemia. Vet. Clin. Pathol. [Internet]. 2008; 37(4):434–437. doi: https://doi.org/bxp8cn

[40] Andrews A. Pregnancy toxaemia in the ewe. In Pract. [Internet]. 1997; 19(6):306–314. doi: https://doi.org/cqddsn

[41] Aly MA, Elshahawy II. Clinico-Biochemical Diagnosis of Pregnancy Toxemia in Ewes with Special Reference to Novel Biomarkers. Alexandria J. Vet. Sci [Internet]. 2016 [cited 12 Feb. 2024]; 48(2):96–102. Available in: https://goo.su/QqnD

[42] Mustafa MK, Saed OS, Ismaeel MA. Clinical and biochemical study of pregnancy toxemia in Iraqi ewes. Arch. Razi. Inst. [Internet]. 2023; 78(3):1131–1139. Available in: https://goo.su/MOTofzK

[43] Constable PD, Hinchcliff KW, Done SH, Grünberg W. Veterinary medicine: a textbook of the diseases of cattle, horses, sheep, pigs and goats. 11th ed. St. Louis (MO, USA): Elsevier Health Science; 2016. 1717 p.

[44] Rebelo-Marques A, Lages A, Andrade R, Ribeiro CF, Mota-Pinto A, Carrilho F, Espregueira-Mendes J. Aging hallmarks: the benefits of physical exercise. Front. Endocrinol. 2018; 9:258 doi: https://doi.org/gdpmcd

[45] Sargison ND, Scott PR, Penny CD, Pirie RS, Kelly JM. Plasma enzymes and metabolites as potential prognostic indices of ovine pregnancy toxaemia—a preliminary study. Br. Vet. J. [Internet]. 1994; 150(3):271–277. doi: https://doi.org/g5rcqv

[46] Holmgren PA, Stigbrand T, Damber MG, Von Schoultz B. Serum levels of placental alkaline phosphatase in high-risk pregnancies. Obstet. Gynecol. 1979; 54(5):631–634. PMID: 503392

[47] Titaux C, Ternynck C, Pauchet M, Stichelbout M, Bizet G, Maboudou P, Onraed B, Clement G, Lenne X, Potier G, Damien S, Chudzinski A. Total alkaline phosphatase levels by gestational age in a large sample of pregnant women. Placenta. [Internet]. 2023; 132:32–37. doi: https://doi.org/g5rcqx

[48] González FHD, da Silva SC. Introdução à bioquímica clínica veterinária. 3th ed. Porto Alegre (Brazil): UFRGS Editora. 2017. 506 p

[49] Kerl ME. Diabetic ketoacidosis: pathophysiology and clinical and laboratory presentation. Compendium [Internet]. 2001 [cited 14 Feb. 2024]; 23(3):220–228. Available in: https://goo.su/ewEMeRW

[50] Oetzel GR. Fresh cow metabolic diseases: Old myths and new data. In: Smith RA, editor [Internet]. Proceedings of the 50th Annual conference of American Association of Bovine Practitioners, Dairy Session; 2017 Sep. 14–16; Omaha (Nebraska, USA); 2017. p. 70–80. doi: https://doi.org/g5rcqz

[51] Petterson JA, Dunshea FR, Ehrhardt RA, Bell AW. Pregnancy and undernutrition alter glucose metabolic responses to insulin in sheep. J. Nutr. [Internet]. 1993; 123(7):1286–1295. doi: https://doi.org/g5rcq3

[52] WeiLi Z, ChangBin X, Lei G,RuiXia X, GuangChao S, Wei Z, ShouRen L, XinHua W, JingQuan Y, JunLiang Y, ShangQuan G. [Cloning and sequence analysis of sheep (Ovis aries) resistin gene (RETN) and its expression pattern in starving model]. J. Agricult. Biotechnol. [Internet]. 2015 [cited 25 Feb. 2024]; 23(8):981–990 Chinese. Available in: https://goo.su/5sUVD

[53] Oliver P, Ribot J, Rodriguez AM, Sanchez J, Pico C, Palou A. Resistin as a putative modulator of insulin action in the daily feeding/fasting rhythm. Pflügers Arch. [Internet]. 2006; 452(3):260–267. doi: https://doi.org/cbd673

[54] Maekawa H, Shioya S, Orita H, Sakurada M, Kushida T, Sato K. Changes of Plasma Levels of Adipocytokines During 120 Hours of Fasting After Endoscopic Treatment. J. Nutr. Disord. Ther. [Internet]. 2017; 7(3):217. doi: https://doi.org/g5rcq6

[55] Nieva-Vazquez A, Pérez-Fuentes R, Torres-Rasgado E, López-López JG, Romero JR. Serum resistin levels are associated with adiposity and insulin sensitivity in obese Hispanic subjects. Metab. Syndr. Relat. Disord. [Internet]. 2014; 12(2):143–148. doi: https://doi.org/g5rcq8

[56] Guo L, Li Q, Wang W, Yu P, Pan H, Li P, Sun Y, Zhang J. Apelin inhibits insulin secretion in pancreatic β-cells by activation of PI3-kinase-phosphodiesterase 3B. Endocr. Res. [Internet]. 2009; 34(4):142–154. doi: https://doi.org/crfzq7

[57] Bertrand C, Valet P, Castan-Laurell I. Apelin and energy metabolism. Front. Physiol. [Internet]. 2015; 6:115. doi: https://doi.org/g5rcrc

[58] Ehrhardt RA, Slepetis RM, Bell AW, Boisclair YR. Maternal leptin is elevated during pregnancy in sheep. Domest. Anim. Endocrinol. [Internet]. 2001; 21(2):85–96. doi: https://doi.org/bdt9d9

[59] Temizel EM, Cihan H, Levent P, Saril A, Özarda Y, Yilmaz Z. Comparison of pre-and postpartum serum leptin, ghrelin, and lipid levels in sheep. Turkish J. Vet. Anim. Sci. [Internet]. 2018; 42(3):177–183. doi: https://doi.org/g5rcrh

[60] Kasimanickam RK. Subclinical pregnancy toxemia-induced gene expression changes in ovine placenta and uterus. Front. Vet. Sci. [Internet]. 2016; 3:69. doi: https://doi.org/g5rcrk

[61] Nørgaard JV, Nielsen MO, Theil PK, Sørensen MT, Safayi S, Sejrsen K. Development of mammary glands of fat sheep submitted to restricted feeding during late pregnancy. Small Rumin. Res. [Internet]. 2008; 76(3):155–165. doi: https://doi.org/dqxhgc

[62] Youssef MA, El-Ashker MR, El-Sayed AA, Ibrahim FA, Awad ME. Hepatic ultrasonography and biochemical alterations in Barki sheep under negative energy balance. Egypt. J. Chem. Environ. Health. [Internet]. 2016; 2(2):265–281. doi: https://doi.org/g5rcrn

[63] Marie M, Findlay PA, Thomas L, Adam CL. Daily patterns of plasma leptin in sheep: effects of photoperiod and food intake. J. Endocrinol. [Internet]. 2001; 170(1):277–286. doi: https://doi.org/c86zkt

[64] Szczesna M, Zieba DA. Phenomenon of leptin resistance in seasonal animals: the failure of leptin action in the brain. Domest. Anim. Endocrinol. [Internet]. 2015; 52:60–70. doi: https://doi.org/f7gcwk

[65] Al-Qudah KM. Oxidant and antioxidant profile of hyperketonemic ewes affected by pregnancy toxemia. Vet. Clin. Pathol. [Internet]. 2011; 40(1):60–65. doi: https://doi.org/crtmvf

[66] Ford EJH, Evans J, Robinson I. Cortisol in pregnancy toxaemia of sheep. Br. Vet. J. [Internet]. 1990; 146(6):539–542. doi: https://doi.org/g5rcrq

[67] Leal-Cerro A, Soto A, Martínez MA, Dieguez C, Casanueva FF. Influence of cortisol status on leptin secretion. Pituitary. [Internet]. 2001; 4(1–2):111–116. doi: https://doi.org/dpgt6c

[68] Weise M, Abad V, Considine RV, Nieman L, Rother KI. Leptin secretion in Cushing's syndrome: preservation of diurnal rhythm and absent response to corticotropin-releasing hormone. J. Clin. Endocrinol. Metab. [Internet]. 1999; 84(6):2075–2079. doi: https://doi.org/g5rcrv