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PATHOHISTOLOGICAL AND BIOCHEMICAL CHANGES IN LACAUNE EWES WITH KETOSIS

Vania Marutsova, Radostin Simeonov

Trakia University, Faculty of Veterinary Medicine, Stara Zagora, Bulgaria
E-mail: vaniamarutsova@abv.bg; radostin.simeonov@trakia-uni.bg

ABSTRACT

The aim of the present study was to establish the pathohistological and biochemical changes in ewes from the Laucane breed with ketosis. Blood samples were obtained from 106 dairy ewes for determination of β-hydroxybutyrate (BHBA), non-esterified fatty acids (NEFA), glucose (Gl), aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT) and total bilirubin (Tb). The ewes were divided in three groups: pregnant, recently lambed and lactating. Target ewes were classified as healthy (C), affected with subclinical ketosis (SCK) and with clinical ketosis (CK) depending on their blood BHBA levels.

The quantities of NEFA in sheep with SCK were statistically significantly elevated, while in sheep with CK – decreased. The levels of glucose decreased, while the activities of ASAT, ALAT and Tb levels were increased in ewes with SCK and CK ketosis. Histological examination revealed cellular vacuolation in hepatocytes, karyolysis, karyorrhexis, necrotic changes and high-grade fatty dystrophy of the liver and kidneys in ewes with ketosis.

Key words: ketosis, β-hydroxybutyrate, enzymes, pathohistological changes, ewes.

Introduction

Pregnancy toxaemia usually occurs in sheep during the last stage of gestation and after lambing (Van Saun, 2000). The disease is the result of inappropriate metabolism of carbohydrates and fats followed by the development of a negative energy balance (NEB) and low levels of glucose in the blood. Predisposing factors for the occurence of the disease are also number of lactations, breed, feeding, the number of fetuses, other diseases (such as lameness or dental disease, that limit food intake), etc. (Hefnawy et al., 2011). Under these conditions, ewes do not meet the nutritional needs of developing fetuses, mobilize more body fat, with resultant ketone body production and hepatic lipidosis (Schlumbohm and Harmeyer, 2008).

The main ketone body in the blood is BHBA. Blood BHBA levels reflect the degree of NEB and lipid mobilization in dairy animals, therefore they are diagnostic markers for SCK and CK (Sordillo and Raphael, 2013). Different blood levels of BHBA have been reported in sheep with SCK – from 0.5 mmol/l to 1.6 mmol/l (Feijó et al., 2015), while in sheep with CK – from 1.6 mmol/l to 7 mmol/l (Balikci et al., 2009). In dairy sheep with pregnancy toxemia, high serum NEFA, low blood glucose and BHBA levels (under 1.0 mmol/l) are reported (Moallem et al., 2015). The glucose is essential for the proper functioning of the brain and its deficiency leads to nervous dysfunction, coma and death.

Non-esterified fatty acids are the other early indicator of the degree of mobilization of fat from body depots. They are a reliable indicator for the evaluation of NEB and the subsequent fatty infiltration of the liver and ketosis (Garverick et al., 2013). The observed uncontrolled mobilization of body fat and the increased accumulation of fatty acids in hepatocytes in animals with ketosis leads to subsequent morphological and physiological changes in the liver (Schlumbohm and Harmeyer, 2008). According to Djokovic et al. (2013) and Simonov and Vlizlo (2014) liver steatosis and hepatocyte degeneration are accompanied with cell membrane damage and release of cytoplasmic

enzymes (ASAT, ALAT and LDH). Balikci et al. (2009) reported high levels of BHBA, ASAT and ALAT in sheep with pregnancy toxaemia.

Van den Top et al. (2005) reported that bilirubinemia is proportional to the accelerated metabolic processes in the liver induced by stress at birth. Djoković et al. (2013) found an increase in total bilirubin in cows with SCK and CK in early lactation due to lower synthesis of enzymes responsible for its elimination. Marutsova and Binev (2020) reported the high levels of bilirubin in goats with SCK.

Kahn and Line (2005) and Radostits et al. (2007) at autopsy of ruminants with CK found extensive fatty infiltration with necrotic lesions of the liver. They reported that the affected liver is enlarged, pale yellow in color and brittle consistency. The bile ducts have degenerative changes and desquamation of the epithelium. The kidneys are soft and pale with degenerative-necrotic changes in the tubules, and the brain is edematized. The lungs are swollen with compensatory emphysema. The adrenal glands are enlarged. In studies of animals with SCK macroscopic changes in the organs are almost undetectable. Clarkson (2000) and Radostits et al. (2007) observed in animals with CK massive lipidosis in hepatocytes, vacuolation in the white matter with the presence of astrocytes and astrogliosis around the vacuolation zones, perivascular mononuclear cell infiltration in the parenchyma and meninges, and neuronal cell necrosis in the brain and cerebellum.

The purpose of the present study was to establish the pathohistological and biochemical changes in ewes from the Laucane breed with subclinical and clinical ketosis.

Materials and methods

Animals

A total of 106 ewes from the dairy breed Lacaune with 200 l annual lactational yield in 2nd and 3rd lactation with average weight 60–80 kg were included in the study.

Experimental design

The sheep were divided into three groups according to their physiological condition: group I – pregnant sheep (between pre-partum days 15 and 0); group II – recently lambed (from postpartum days 0 to 15) and group III – lactating (from postpartum days 30 to 45). Blood chemical analysis of BHBA concentrations was performed in all sheep in order to classify them as control (C, BHBA <0.8 mmol/l), affected with SCK (BHBA from 0.8 to 1.6 mmol/l) and CK (BHBA >1.6 mmol/l). The different groups of sheep were reared under equal conditions and fed according to their physiological status.

Blood samples and analyses

Blood samples were collected through puncture of the jugular vein using sterile 21G needles and vacutainers without anticoagulant and with heparin (5 ml, Biomed, Bulgaria). Samples were obtained in the morning before feeding and were stored and transported at 4^{0}C. Analysis was performed within 24 hours after sampling. Blood BHBA and glucose concentrations were determined in situ using a portable Xpress-I system (Nova Biomedical, UK). The following indices were determined: non-esterified fatty acids (NEFA, mmol/l), aspartate aminotransferase (ASAT, U/l), alanine aminotransferase (ALAT, U/l) and total bilirubin (Tb, μmol/l). Biochemical paramaters were measured using an automated biochemical analyser Mindray BS-120 (China). The values of NEFA in the blood serum were determined using NEFA ELISA Kit (Crystal Day Biotech Co., LTD., China) and ELISA Reader Sunrise (Tecan, Switzerland). After performing a complete autopsy on dead sheep (n = 6) with a CK, liver and kidney material was taken for histological examination. They were fixed

in 10% neutral formalin and processed by routine histological technique (Dzhurov et al.,1989). Samples were included in paraffin blocks cut on a microtome (slice thickness 4 μm) and stained with hematoxylin-eosin (H&E).

Statistical analysis

Statistical analysis was done with Statistica 6.0, StatSoft, Inc. (USA, 1993) and ANOVA test. Results were presented as mean (x) ± standard deviation (SD). The level of statistically significance was $p<0.05$.

Results

The results from the biochemical analysis in the three groups of Lacaune sheep are shown in Table 1.

Blood BHBA analysis in the three control groups of Lacaune sheep were within the reference range (Table 1). Sheep from the group I, II and III with SCK had statistically significantly higher BHBA than controls ($p<0.001$). Sheep from groups I, II and III with CK had BHBA levels in blood substantially higher than both controls and SCK ($p<0.001$) (Table 1).

Blood serum NEFA concentrations of the three control groups ranged within the reference interval (Table 1). In the groups I, II and III with SCK, blood NEFA values were statistically significantly higher than controls ($p<0.05$). All sheep with CK exhibited lower blood NEFA concentrations than those with SCK ($p<0.05$) (Table 1).

Blood glucose levels in the three control groups of ewes were within the physiological range (Table 1). In sheep from three groups with SCK and CK its values decreased significantly compared to those of the control groups ($p<0.05$; $p<0.001$) (Table 1).

In the three groups of ewes the blood activity of ASAT and ALAT varied close to physiological range (Table 1). The blood activity of ASAT and ALAT in ewes from group I, II and III with SCK increased statistically significantly vs control values ($p<0.05$, $p<0.01$). The activities of ASAT and ALAT in ewes from the three groups with CK was substantially higher than respective controls (Table 1).

The changes in the levels of total bilirubin in sheep from the control groups are presented in Table 1, which shows that its values vary around the physiological norms. In ewes from groups with SCK and CK its values increased statistically significantly compared to control groups ($p<0.01$; $p<0.001$) (Table 1).

Table 1: Changes in blood biochemical parameters in Lacaune ewes from I, II and III groups SCK and CK (mean ± SD).

Parameters / Groups	C (control)			SCK (subclinical ketosis)			CK (clinical ketosis)		
	I (15–0 days)	II (0–15 days)	III (30–45 days)	I (15–0 days)	II (0–15 days)	III (30–45 days)	I (15–0 days)	II (0–15 days)	III (30–45 days)
BHBA (mmol/l)	0.51±0.15	0.51±0.12	0.40±0.10	1.11±0.24^{1c}	1.20±0.28^{1c}	1.07±0.24^{1c}	3.10±0.60^{1c}	2.15±0.63^{1c}	2.26±0.23^{1c}
NEFA (mmol/l)	0.65±0.02	0.47±0.26	0.21±0.04	1.02±0.05^{1a}	0.69±0.04^{1a}	0.45±0.05^{1a}	0.71±0.01	0.42±0.06	0.36±0.01
Glucose(mmol/l)	3.30±0.60	3.47±0.52	3.38±0.53	2.36±0.61^{1a}	2.35±0.64^{1a}	2.62±0.29^{1c}	2.26±0.77^{1a}	2.32±0.31^{1a}	2.37±0.63^{1a}
ASAT (U/L)	58.2±19.4	43.0±8.6	51.0±12.3	134.7±28.4^{1b}	139.1±25.9^{1c}	163.6±20.9^{1c}	142.0±20.6^{1b}	154.0±33.5^{1c}	168.6±20.2^{1c}
ALAT (U/L)	20.6±5.0	27.3±6.5	23.0±4.1	41.2±4.0^{1a}	63.0±6.2^{1b}	58.3±2.5^{1b}	54.0±12.1^{1a}	59.5±8.2^{1b}	68.3±2.5^{1c}
Tb (µmol/l)	4.67±0.1	4.84±0.2	4.9±0.8	5.98±0.3^{1b}	6.4±0.8^{1b}	6.36±0.9^{1c}	7.46±0.7^{1c}	6.6±0.9^{1b}	7.3±0.9^{1c}

Legend: $^{a}p<0.05$; $^{b}p<0.01$; $^{c}p<0.001$; 1-vs control groups

Macromorphological changes were observed mainly in the liver and kidneys of autopsied sheep. The liver was enlarged, with rounded edges and with yellow-orange color. Its consistency was brittle (Fig. 1). The kidneys were slightly enlarged, with a soft consistency and a lack of a clear boundary between the cortex and the medulla (Fig. 2). The smell of ammonia was felt when the sheep's rumen was opened and hemorrhagic enteritis was observed.

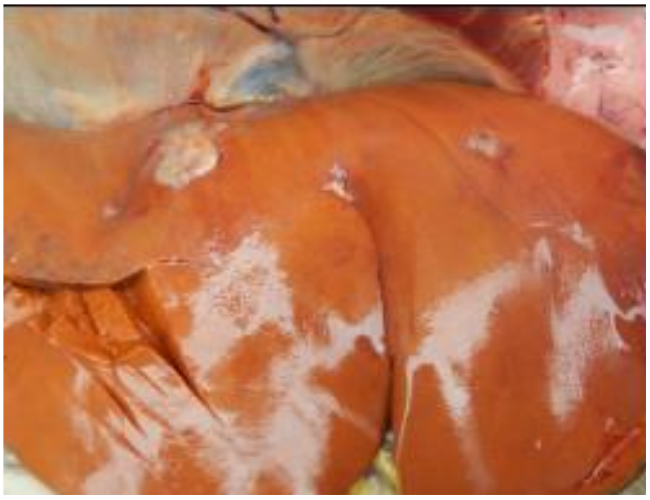

Figure 1: Liver of Lacaune ewe with clinical ketosis.

Fiure 2: Kidney of Lacaune ewe with clinical ketosis.

Pathohistological examinations of the liver of sheep with a clinical form of ketosis are such that in the cytoplasm of liver cells were observed many optically empty bubbles (vacuoles), which were the extracted fats during the process of the preparation (Fig. 3). At this stage, hepatocyte nuclei did not show abnormalities.

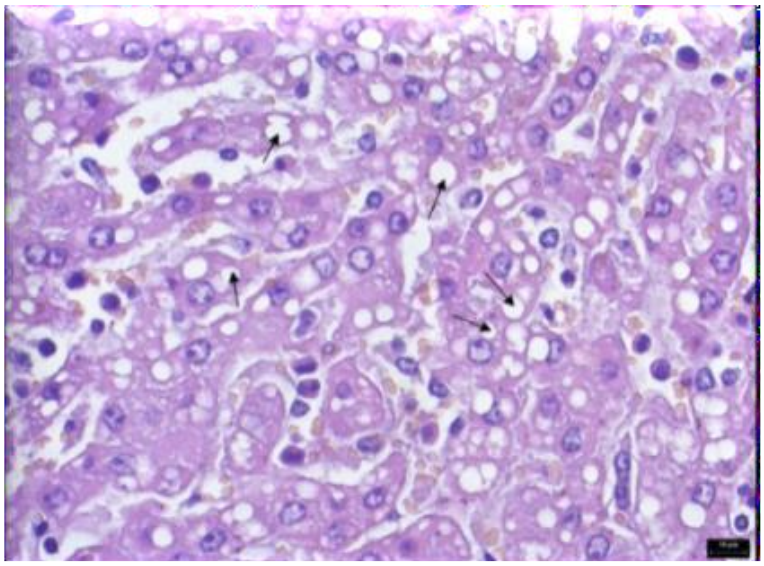

Figure 3: Liver of Lacaune ewe with clinical ketosis. Fat infiltration. Staining with H&E.

Activated Kupffer cells and mononuclear proliferates were visualized in some areas against the background of hyperemic blood vessels (Fig. 4).

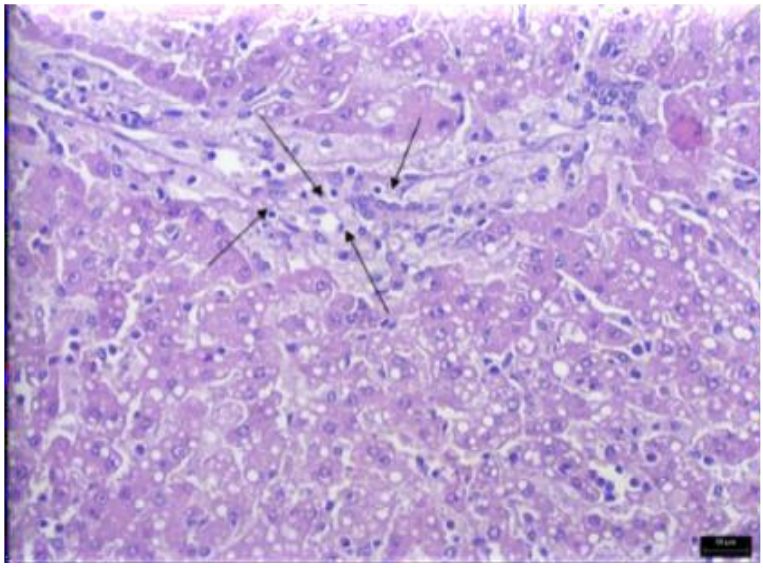

Figure 4: Liver of Lacaune ewe with clinical ketosis. Activated Kupffer cells and mononuclear proliferates. Fat infiltration. Staining with H&E.

In the terminal stage of the disease, the periphery of the liver particles was lighter in color. High-grade fatty degeneration, karyolysis, and karyorrhexis were observed in the cells. Necrobiotic changes were found in many liver cells, which masked the normal liver structure. Macrophages, erythrocytes, bile and blood pigments were visualized between the decayed cells. The capillaries were hyperemic, and in some areas – with a destroyed wall (Fig. 5).

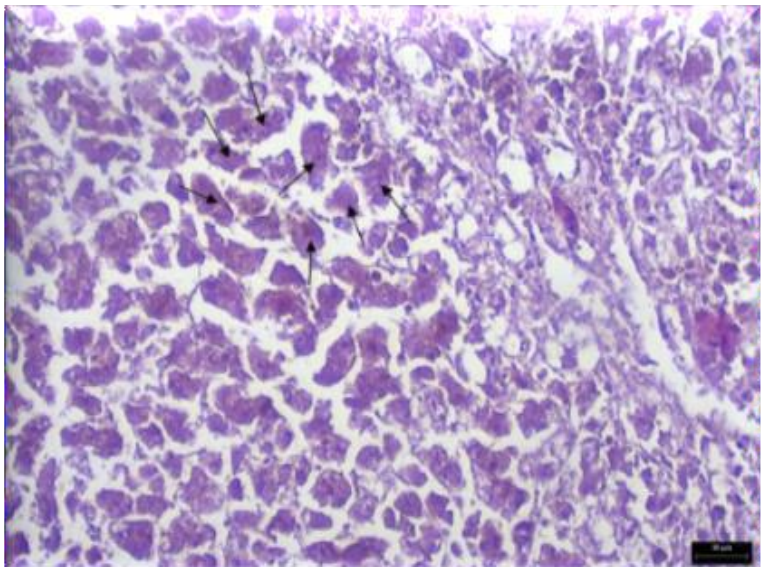

Figure 5: Liver of Lacaune ewe with clinical ketosis. High-grade fatty degeneration, karyolysis, karyorrhexis and necrobiotic changes in hepatocytes. Staining with H&E.

In the final stages of the disease, fatty infiltration was observed in the kidneys, followed by granular and fatty dystrophy. In some areas, epithelial cells fell out of the basement membrane into the renal tubules, and in others, lymphocytes and histiocytes accumulated (Fig. 6).

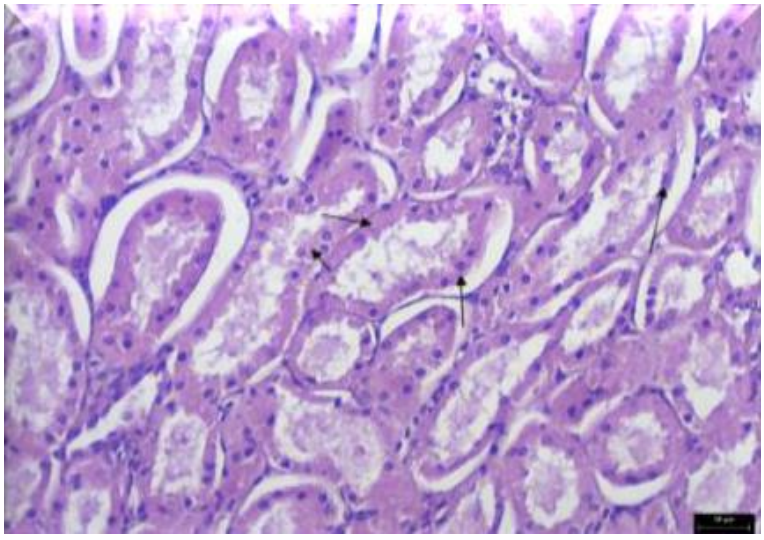

Figure 6: Kidney of Lacaune ewe with clinical ketosis. Parenchymal dystrophy. Staining with H&E.

Discussion

Pregnancy toxemia is a metabolic disease in ewes during late gestation and early lactation, characterized by low morbidity and high mortality. It occurs as a result of increased energy needs, accompanied by insufficient or inadequate nutrition (Schlumbohm and Harmeyer, 2008). The sensitivity of ewes to ketosis becomes higher during the last four to three weeks of gestation as a result

of rapid growth of the fetus and increased needs of glucose (Kaneko et al., 2008). It is known that 80% of fetal growth occurs during the last 6 weeks of gestation, utilizing 40% of maternal glucose (Kaneko et al., 2008). The reduced blood glucose concentration leads to increased mobilization of lipids from the adipose tissue and stimulation of ketogenesis for maintenance of metabolic homeostasis (Moghaddam and Hassanpour, 2008). BHBA in ruminats is synthesized both by ketogenesis and in the rumen by butyrate-producing bacteria. Increased levels of BHBA in blood during ketosis are a response to occurring carbohydrate deficiency and tricarboxylic acid cycle inhibition (Reece, 2004). High levels of this ketone body induce hepatic oxidative stress, apoptosis and inflammation (Song et al., 2016). In our research we found that the sheep from the dairy breed Lacaune suffered from SCK and CK during the late pregnancy, lambing and lactation. Blood BHBA levels reached 1.20 mmol/l in animals with SCK and 3.10 mmol/l in ewes with CK. Our data are similar to those of Balikci et al. (2009) and Anoushepour et al. (2014). The rate of ketone body formation is directly proportional to the degree of lipolysis and oxidation of fatty acids (Roche et al., 2013).

The observed changes in blood NEFA concentrations as indicator of NEB were not unidirectional: in dairy ewes with SCK, NEFA levels were increased vs controls. These results prove the thesis that the lipolysis in the period of lactation proceeds rapidly so that the large quantity of NEFA can not be metabolized in the liver (Roche et al., 2013). In the three groups of ewes with CK, NEFA levels decreased. This is due to the fact that fatty acids that are not completely oxidized are either converted into ketone bodies or are reesterified to triglycerides (Holtenius and Holtenius, 1996).

The glucose is an important metabolic fuel for many organs and systems in the dairy animal. Some vital cells (erythrocytes, brain and kidney cells) rely on glucose as the sole energy substrate (Aschenbach et al., 2011). In our studies, we found that the amount of glucose in the blood of sheep with SCK and CK decreased significantly. The glucose metabolism in ruminants is regulated by various hormones, depending on the degree of uptake and realization by food, as well as the available glucose precursors. The amount of glucose precursors varies depending on the stages of lactation, food intake, adipose tissue mobilization and energy balance. Volatile fatty acids produced in the rumen are the main precursors for gluconeogenesis in the liver (50-60%) (Sordillo and Raphael, 2013). Another important source of glucose is glycogen. Stored in the liver or skeletal muscle, it directly and indirectly maintains glucose levels in the body (Kuhla et al., 2011). According to Schlumbohm and Harmeyer (2008), decreased glucose production, as a result of stress in the last weeks of pregnancy in sheep carrying twins is the main cause of hypoglycaemia. Decreased ability of sheep to use BHBA at the end of pregnancy and decreased metabolic efficiency of the liver are considered to be the main causes of hyperketonemia (Harmeyer and Schlumbohm, 2006).

The liver enzymes ASAT and ALAT in Lacaune ewes included in the study showed increased activity in animals with SCK and CK. A positive correlation between blood BHBA levels and ASAT was found out in the affected animals. ALAT is a very specific enzyme for hepatocyte damage. The increased activity of transaminases occurred in response of liver parenchyma damage, leading to their release of cellular organelles in the blood (Cal et al., 2009). Elevated serum levels of ASAT and gamma-glutamyl transferase (GGT) are used as indicators of liver injury in dairy animals with ketosis, and correlate with the degree of histological changes found by us in cows and sheep with both forms of ketosis. High levels of liver enzymes are the result of liver lipidosis, cholestasis or abnormal hepatobiliary circulation. Our data are comparable to those reported by Balikci et al., (2009). Contrary to our findings, Anoushepour et al., (2014) did not observed any significant changes in the blood activity of these two liver enzymes.

Our studies show, that the amount of bilirubin in the blood increased in ewes with SCK and CK. Well-defined bilirubinnemia was observed in pregnant sheep with a CK, reaching values of 5.98 μmol/l, followed by lactating sheep. The same tendency of change is found in the groups of animals with SCK. Impaired pigment function of the liver would be attributed to the dystrophic changes in it, which mechanically prevent the release of bilirubin in the bile. That results in increasing of the bilirubin in the blood. Similar views have been expressed by other authors (Van den Top et al., 2005; Djoković et al., 2013), unlike Ferris (1969) who did not found any changes in blood bilirubin levels in sheep with pregnancy toxaemia. The established macroscopic changes of the organs (liver – enlarged, with yellow-orange color and brittle consistency; kidney – enlarged and soft; rumen – smell of ammonia; intestines – hemorrhagic enteritis) at autopsy of sheep with CK are indicative of the diagnosis. Our data are comparable to the one reported by Kahn and Line (2005) and Radostits et al. (2007).

Pathohistological changes in the liver and kidneys (fatty dystrophy, karyolysis, karyopyknosis, fatty infiltration) are evidence of impaired metabolic function of the liver (pigment, carbohydrate, enzyme, etc.), established by us by examination of relevant biochemical parameters. Our data are comparable to those reported by Clarkson (2000) and Radostits et al. (2007). Sigh et al. (1995) found degeneration and hyalinization of cardiac myofibrils. Ferris et al. (1969) found structural changes in the renal glomeruli, while McCausland and O'Hara (2004) did not find structural changes in the glomeruli.

Conclusion

The monitoring of SCK and CK in sheep by measuring BHBA and NEFA is necessary to become a mandatory preventive measure. Blood biochemical analysis in ewes with hyperketonemia present hyperenzymemia, bilirubinemia and hypoglycemia. Histological preparation revealed cellular vacuolation in hepatocytes, caryolysis, karyorrhexis, necrotic changes and high-grade fatty dystrophy of the liver and kidneys in ewes with ketosis. Timely diagnosis of the ketosis at the herd level and taking corrective actions related to the adjustment of the ration will prevent the occurrence of permanent morphological damage to the organs.

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