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Dynamics of some blood biochemical parameters in Boujaâd ewes from early to late gestation and the possibility of early pregnancy diagnosis

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Abstract. *This study aimed to evaluate the biochemical parameters of Boujaâd sheep under Moroccan semi-arid conditions during pregnancy and postpartum stages in pregnant (P) ewes compared to non-pregnant ewes (NP). Blood samples were collected from 24 healthy Boujaâd ewes (59 ± 4.2 kg) enrolled in the current study. From (1 to 30 days), these samples were collected daily; from day 30 onwards, they were gathered at three-day intervals. Blood samples were taken from the jugular vein to be analyzed for glucose, cholesterol, total protein, creatinine, urea, and triglycerides. In the present study, no significant difference was observed before synchronization in all parameter studies between pregnant ewes (P) and non-pregnant ewes (NP) (P>0.05). The results showed the lowest glucose level in pregnant ewes during all pregnancy stages, while total proteins, urea, and triglycerides showed the opposite trend. During early pregnancy (18-30 days), the cholesterol levels were lower in P compared with NP ewes, whereas no difference was found from 30 days to parturition. Creatinine was higher in pregnant ewes during early pregnancy (18-30 days). In comparison, there was no statistically significant (P>0.05) variation in creatinine levels between the P and NP ewes after 30 days of gestation. To conclude, marked changes accompanied specific biochemical parameters. The biochemical parameter indicating a clear difference is triglycerides, which may indicate an early pregnancy diagnosis in sheep considering the exact day of mating or artificial insemination in animals.*

Keywords: blood biochemical parameters, ewes, pregnancy stages, early pregnancy diagnosis

Introduction

There is no doubt that small ruminant production is a significant component of livestock production worldwide, especially in developing countries where it is often the principal source of wealth and high-quality nutrition in animal protein. Indeed, it is essential for both economic and social domains in terms of the income they provide. However, this farming is managed traditionally in most areas and farms, where it is undergoing climatic, nutritional, and pathological hazards (Swanson et al., 2004; Yokus et al., 2006). Consequently, low herd production is attributed

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to poor breeding and herd-feeding behavior, which is often extensive (Bencherif, 2011). Successful breeding is a significant factor contributing to the efficiency of milk and meat production (Khanum et al., 2008). Therefore, a good understanding of physiology during the various stages of the animal's reproduction is necessary. The challenge for animal production is to produce more animal protein to feed a growing population while reducing its environmental footprint (Rockström et al., 2017; Chang et al., 2021).

One of the most effective fertility management strategies to prevent and reduce economic losses in dairy herds is identifying pregnant and non-pregnant animals as soon as possible after insemination (Ott et al., 2014). Therefore, there is a need for early pregnancy diagnosis in small ruminants. Indeed, maintaining animals' inadequate nutritional status, timely insemination, and early detection of pregnancy status are three basics for effective fertility management (Ott et al., 2014; Northrop et al., 2019). Early detection of pregnancy status following mating or insemination allows for critical management decisions for lactating females to ensure they remain in the herd/flock and are productive (Bekele, 2016). Many techniques have been used to detect early pregnancy in sheep, such as transrectal ultrasonography, radioimmunoassay (RIA), and ELISA comprises the quantification of steroid hormone and glycoprotein concentrations in blood and milk samples but is more time-consuming and expensive, and requires a more expert operator (Karen et al., 2003; Barbato et al., 2009; Gonzalez-Bulnes et al., 2010). To avoid such a problem, analysis of biochemical parameters could be an alternative to identify pregnancy-associated glycoprotein (PAG) concentrations or steroid hormones in the blood sample. For that reason, determining metabolic blood profiles using plasma biochemical parameters is necessary to study ruminant metabolic changes in various reproduction phases (Balıkcı et al., 2007; Shwetha et al., 2018). As far as we know, no study has yet examined biochemical parameters on daily samples starting on day 18 following mating to identify pregnancy in ewes and perform an early pregnancy diagnosis in the ewe.

Therefore, this study was made to investigate blood metabolite concentrations as an indicator of the early pregnancy diagnosis in ewes from 18 days (pre-mating) and to evaluate the impact of pregnancy stages (from early to late pregnancy) and postpartum on blood biochemical parameters.

Material and methods

Animals and management

All the experimental procedures involving animals in this study were conducted by the Institutional Animal care guidelines of the National Institute of Agronomic Research (INRA), Morocco, and approved by the Administration Committee of Experimental Animals (INRA, Morocco) (number: 01/CRRAT/2017).

A total of 24 healthy Boujaâd ewes, multiparous and primiparous, aged between 2 and 4 years and weighing 59 ± 4.2 kg, were used in the present study. Animals were kept semi-arid at Morocco's National Institute of Agricultural Research in Settat (32°95.455'N, -7°62.566'W). The animals were kept under the same food, housing, and lighting settings. Lighting varied with the day's length. The temperature ranged between 28°C during the day and 15°C at night. All animals were housed indoors in a covered shelter (500 m^2) and were free to roam. Ewes were given a combination of straw–hay, barley, and sunflower. The daily amounts offered per head were 1000 g of grass, 800 g of barley, and 300 g of sunflower meal (grain). The animals were divided into two groups: eight (8) non-pregnant ewes (NP) and 16 pregnant ewes (P).

Estrous (heat) synchronization and blood sampling

The ewes were synchronized using intravaginal sponges (Chronogest, Laboratorios Intervet International B.V., Boxmeer, The Netherlands) and impregnated with 20 mg of fluorogestone acetate. After 14 days, the sponges were removed, and the ewes received 300 IU of PMSG (Folligon, Pregnant Mare Plasma Gonadotropins) intramuscularly.

Before synchronization, blood samples were collected on day 0 (the day of pre-mating). All ewes were naturally mated with rams 48 hours after sponge removal. The blood sample was carried out in three periods: 1/ The first collection was daily from day 0 to day 50. 2/ The second samples were collected every three days throughout the pregnancy until lambing (day 51 to day 150). 3/ The last

samples were taken daily, corresponding to the postpartum (day 151 to day 170). Blood samples were collected in vacuum Venoject® tubes (Sterile Terumo Europe, Leuven, Belgium) from the jugular vein; immediately after collection, samples were transported to the laboratory and centrifuged at 2000 x g for 10 min. The plasma samples were stored at −20°C until the assessment. Pregnancy was diagnosed in the second month after synchronization by ultrasound scanning by using a real-time ultrasound scanner equipped with a 3.5 MHz linear-array transducer (Aloka SSD-500, Aloka Co. Ltd., Tokyo, Japan), establishing at this moment two groups: NP (n = 8) and P (n = 16).

Blood plasma analysis

The whole blood was collected into anticoagulant-treated tubes (EDTA-treated). Cells were removed from the plasma by centrifugation for 10 minutes at 1,000-2,000 x g using a refrigerated centrifuge.

To determine the biochemical parameters (glucose, cholesterol, triglycerides, urea, total proteins, and creatinine), an automated analyzer (Autolyser AL 820, Swiss) was used. Plasma glucose levels were measured using the GOD-POD method based on the glucose being oxidized by the enzyme glucose oxidase (GOD) to gluconic acid and hydrogen peroxide (H_2O_2). Thus, formed H_2O_2 oxidatively couples with 4-amino antipyrine and phenol in the presence of peroxidase (POD) to develop red-colored quinone imine dye, measured at 540 nm. The intensity of the color is directly proportional to the glucose concentration in the sample.

Plasma cholesterol concentration was calibrated calorimetrically as described by Watson (1960). Plasma triglycerides were measured enzymatically using the GPO-POD method based on the enzymatic determination of glycerol using the enzyme phosphate oxidase (GPO) after hydrolysis by lipoprotein lipase. Total plasma protein levels were determined via the Lowry method, the principle of which is based on the Alkaline CuSo4 that catalyzes the oxidation of aromatic amino acids with subsequent reduction of sodium potassium molybdate tungstate of Folin's reagent, giving a purple color complex. The intensity of the color is a direct proposition to the concentration of the aromatic amino acid in the given sample solution (Lowry et al., 1951). Plasma urea was assayed by the Berthelot reaction for measuring the ammonia released by the enzymatic action of urease, and plasma creatinine was determined by the Jaffe reaction method described by Seaton and Ali (1984).

Statistical analysis

Statistical analyses were performed using the JMP SAS 11.0.0 (SAS Institute Inc., Cory, NC, USA) program. The data obtained were first tested for normality and homogeneity using the Shapiro–Wilk and Kolmogorov–Smirnov tests, respectively. The data of blood plasma composition were analyzed by a factorial design ANOVA (one-way). Tukey's post hoc test was applied to compare the means at $P<0.05$ for the effect of the pregnancy period. The student's t-test was used to compare the means at $P<0.05$ between pregnant and non-pregnant ewes at each pregnancy period. The results were expressed as mean ± standard error.

Results

The results of plasma biochemical analysis in pregnant and non-pregnant ewes (P and NP) during pregnancy stages are presented in Table 1. Our findings revealed no significant differences in the parameters studied between P and NP sheep before gestation. Furthermore, plasma glucose concentrations were lower in the P ewes compared to the NP ewes during all pregnancy periods. There was a statistically significant ($P<0.05$) decrease in the glucose level from early to late gestation periods. In contrast, total protein, urea, and triglyceride levels were higher in all pregnancy periods in P than NP ewes. In addition, our data showed that cholesterol concentration was significantly lower in pregnant ewes for 18-30 days, while no significant difference was found between P and NP ewes from 30 to late gestation. Creatinine level was higher in P than NP ewes during pregnancy (1-30 days). However, no difference ($P>0.05$) was recorded in creatinine between P and NP ewes from 30 days to late gestation. During the postpartum period, plasma glucose, cholesterol, and creatinine levels were lower in the N group compared to the NP one ($P>0.05$). Contrary to the plasma, total protein, urea, and triglycerides levels were higher in the N vs. NP group ($P<0.05$).

Table 1. Mean (±S.E.) (S. E= Standard error) of blood metabolite levels of pregnant ewes (n = 16) at Days 0 (pre-mating); 01-18, 18-30, 30-50, 50-100, 100-150 periods of pregnancy and 20 days postpartum compared to non-pregnant ewes (n = 8).

Parameters	Pregnancy period (days)						Post-partum period (days)
	0	01–18	18–30	30–50	50–100	100–150	150–170
Glucose (mmol/L)							
Non-pregnant	3.3 ±0.01^{Aa}	3.35 ±0.01^{Aa}	3.52 ±0.01^{Aa}	3.24 ±0.02^{ABa}	3.13 ±0.03^{ABa}	2.91 ±0.02^{Ba}	3.19 ±0.02^{Ba}
Pregnant	3.19±0.01^{Aa}	3.24 ±0.01^{Aa}	3.31±0.01^{Ab}	2.91 ±0.01^{ABb}	2.75 ±0.01^{Bb}	2.58 ±0.02^{Bb}	2.91±0.01^{ABb}
Cholesterol (mmol/L)							
Non-pregnant	1.52 ±0.01^{Aa}	1.57 ±0.01^{Aa}	1.57 ±0.01^{Aa}	1.68 ±0.08^{Aa}	1.62 ±0.02^{Aa}	1.62 ±0.04^{Ab}	1.60 ±0.04^{Aa}
Pregnant	1.47 ±0.01^{BCa}	1.52 ±0.01^{Aa}	1.34 ±0.01^{Cb}	1.49 ±0.01^{BCb}	1.65 ±0.01^{Ba}	1.81 ±0.02^{Aa}	1.37 ±0.01^{Cb}
Total protein (g/L)							
Non-pregnant	64.91±0.83^{Aa}	69.01±0.6^{Aa}	58.60±0.52^{Bb}	56.75±1.07^{Bb}	56.47±3.82^{Bb}	56.95±3.02^{Bb}	55.26±3.14^{Bb}
Pregnant	68.59±2.01^{Ba}	70.82±0.86^{Ba}	68.25±0.67^{Ba}	63.15±1.79^{Ca}	59.77±0.54^{Ca}	60.79±0.58^{Ca}	75.90±1.98^{Aa}
Creatinine (µmol/L)							
Non-pregnant	92.82±0.62^{Aa}	88.31±0.17^{Aa}	90.69±0.09^{Aa}	87.25±0.21^{ABb}	71.78±0.31^{Cb}	78.14±0.28^{BCa}	72.57 ±0.25^{Ca}
Pregnant	93.52±0.54^{Aa}	77.61±0.43^{Ca}	85.04±0.17^{Ca}	95.56±0.11^{ABa}	100.33±0.24^{Aa}	88.04 ±0.48^{BCa}	69.65 ±0.30^{Da}
Urea (mmol/L)							
Non-pregnant	3.3 ±0.02^{Aa}	4.8 ±0.01^{Aa}	4.2 ±0.01^{Aa}	4.0 ±0.02^{Aa}	3.7 ±0.02^{Ab}	3.7 ±0.02^{Ab}	3.7 ±0.01^{Aa}
Pregnant	3.8 ±0.01^{Da}	4.5 ±0.01^{Da}	4.3 ±0.01^{Da}	3.7 ±0.01^{Da}	5.6 ±0.01^{Ca}	8.5 ±0.01^{Aa}	6.5 ±0.01^{Bb}
Triglycerides(mmol/L)							
Non-pregnant	0.61±0.04^{Aa}	0.58±0.05^{Aa}	0.65±0.04^{Ab}	0.58 ±0.03^{Ab}	0.54±0.02^{Bb}	0.49±0.03^{Bb}	0.54±0.01^{Bb}
Pregnant	0.56±0.08^{Ba}	0.67±0.04^{Aa}	0.72±0.03^{Aa}	0.68±0.03^{Aa}	0.56 ±0.01^{Ba}	0.49±0.01^{BCa}	0.42±0.01^{Ca}

Means in the same row and within the same variable with different superscripts differ significantly ($P < 0.05$).

A, B, C: Different superscripts within lines indicate an effect of the pregnancy period on Boujaâd ewes blood compounds in both pregnant and non-pregnant ewes at ($P<0.05$) using Tukey's post hoc test.

a, b, c: Different superscripts within columns indicate an effect of the experimental group (pregnant vs. non-pregnant ewes) at each pregnancy period at ($P<0.05$) using the student's t-test.

The profile of biochemical parameters (from 0-30 days) for pregnant (P) and non-pregnant (NP) ewes are summarized in Figure 1. Plasma glucose, cholesterol, and creatinine levels were significantly lower in pregnant than non-pregnant ewes. Total protein levels were significantly higher in pregnant ewes, while both groups recorded a non-significant difference in plasma urea levels. Surprisingly, a significant level of triglycerides was recorded in pregnant ewes from 20 to 30 days after mating.

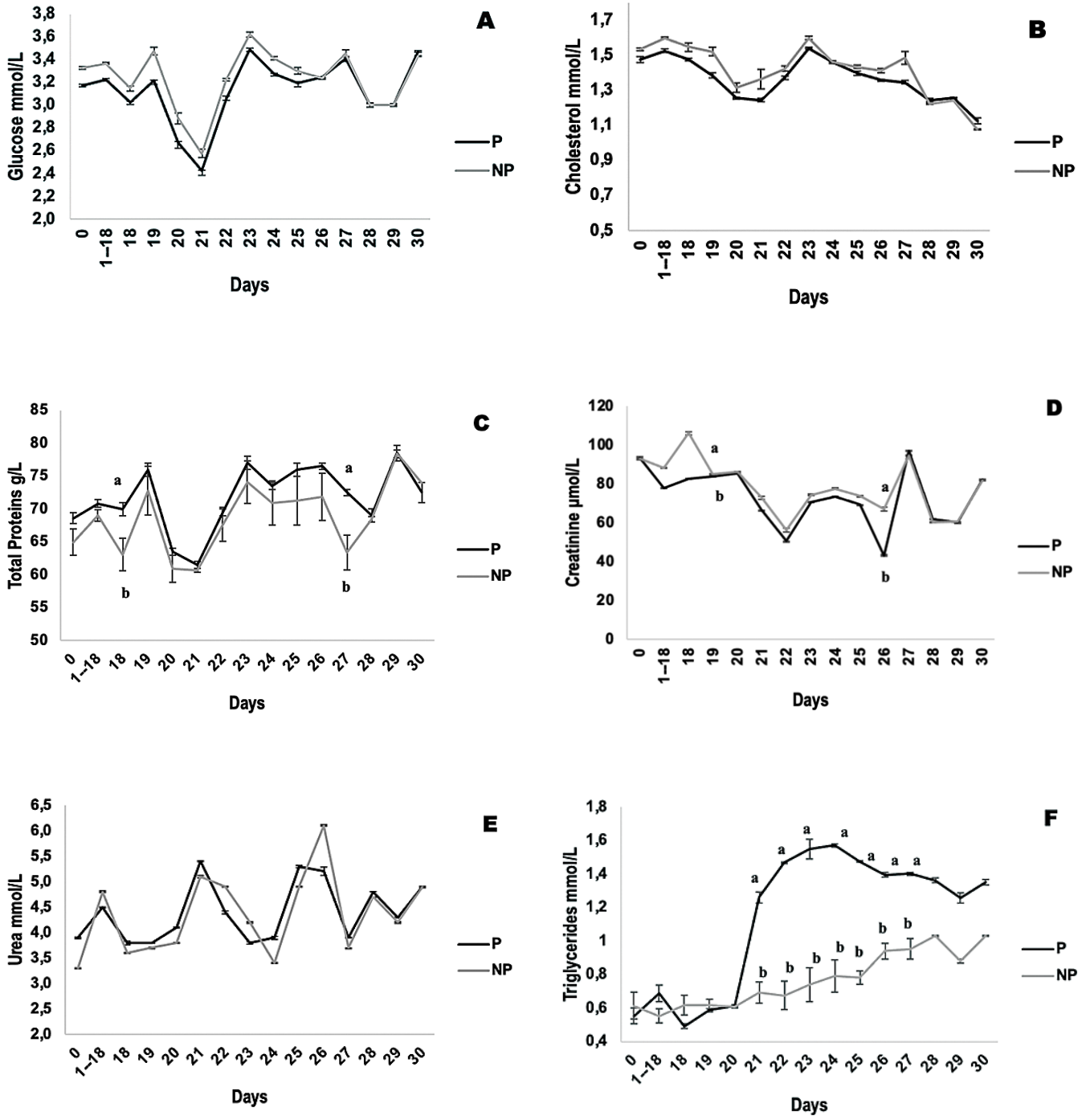

Figure 1. Biochemical parameters for pregnant (P) and non-pregnant (NP) ewes for samples obtained from the day 0 to day 30 after mating (a zoom on the first period of gestation day by day from 18 to 30 days). A/ Glucose (mmol/L), B/ Cholesterol (mmol/L), C/ Total protein (g/l), D/ Creatinine (µmol/L), E/ Urea (mmol/L), F/ Triglycerides (mmol/L).

a,b a letter indicates a significant difference between pregnant and non-pregnant ewes during early pregnancy ($P < 0.05$)using the student's t-test.

Discussion

In sheep farming, effective monitoring of the herd implies reasonable reproduction control. This study aims to investigate the variation in blood plasma levels during pregnancy and postpartum stages in Boujaâd sheep bred in semi-arid Moroccan environments. To guarantee that these results are exclusively related to the location of gestation, the environmental conditions and nutrition were matched for all the ewes in this study. Because of changes in physiology, there are significant changes in the blood biochemical parameters of animals before and after parturition (Gürgöze et al., 2009; Akbulut et al., 2021). The present study showed that triglycerides might indicate an early pregnancy diagnosis in Boujaâd ewes. More precisely, higher plasma triglyceride concentrations were observed from 18 to 50 days of pregnancy and decreased significantly after 50 days. As a result, increased blood triglyceride levels may result from energy mobilization from adipose tissue, particularly in energy-deficient animals associated with pregnancy. (Oliveira et al., 2014). On the other hand, the number of total insulin receptors decreases with advancing gestation, which could lead to reduced triglyceride concentration due to ineffective stimulation of lipogenesis by insulin (Yokus et al., 2006). According to Piccione et al. (2009), a gradual decline in plasma triglyceride levels was seen in lactating ewes during the postpartum period due to an increase in the concentration of these compounds in the ewes' liver. The significant decrease in plasma triglycerides in the post-partum period could be explained as increased lipolysis, which is hormonally regulated (Holtenius and Hjort, 1990). Pesántez-Pacheco et al. (2019) found that Maternal plasma concentrations of triglycerides and cholesterol increased during pregnancy. Serum triglyceride levels were also above the upper range reported by Kaneko et al. (2008) in Akkaraman ewes at 150 days gestation (Balıkcı et al., 2007) and Ouled djellal ewes (Deghnouche et al., 2013). In contrast, Kandiel et al., (2016), reported lower serum triglyceride concentration in mid-pregnancy Barki ewes. Differences in serum triglyceride levels between parturition and 30th day postpartum were significant in twin births (Akbulut et al., 2021).

Glucose is the primary metabolic fuel used by the fetus of sheep (Haffaf and Benallou, 2016). Moghaddam and Hassanpour (2008) and Takarkhede et al. (1999) noted that plasma glucose concentrations were higher during lactation than during pregnancy in ewes. Henze et al. (1994) and Karapehlivan et al. (2007) observed higher blood glucose levels in P ewes. In contrast, Firat A and Ozpinar A (2002) did not record significant changes in plasma glucose levels during pregnancy. In 2007, Balikci et al. reported that serum glucose levels were lower on days 100 and 150 of pregnancy compared to 45 days postpartum. This study recorded lower glucose levels in P than NP ewes during all pregnancy stages and 20 days postpartum. This finding can be explained by the increase in maternal permeability to glucose and its consumption by the fetus during pregnancy (Tontis and Zwahlen, 1987; Sahlu et al., 1995; Deghnouche et al., 2013) and the production of milk (Ramin et al., 2007; Kaneko et al., 2008). The lower plasma glucose levels in pregnant ewes are also attributed to impaired gluconeogenesis by the liver from glucogenic precursors such as propionate, which is derived from rumen fermentation (Dzadzovski et al., 2015). In contrast, the increased plasma glucose levels during the postpartum period must be considered because of its mobilization for milk lactose synthesis (Brozostowski et al., 1995).

The change in total proteins during the progression of pregnancy was reported in a different study with a contradictory result. Yokus et al. (2006) found that total protein concentration in sheep did not change with reproductive status, while Balikci et al. (2007) and Brozostowski et al. (1995) reported that it decreased by day 150 of gestation in ewes. Likewise, our results revealed significantly lower levels from day 30 of pregnancy. This decrease during pregnancy could be explained by the fact that the fetus synthesizes all its proteins from amino acids derived from the mother, and the growth of the fetus increases exponentially, reaching a maximum level, especially in the muscles during late pregnancy (Jainudee and Hafez, 1994; Antunović et al., 2002). Besides, El-Sherif and Assad (2001) and Brozostowski et al. (1995) reported that plasma total protein decreases during late pregnancy, which could be explained by the extraction of plasma immunoglobulin during the last period of gestation for colostrum synthesis (Kaneko et al., 2008; Braun et al., 2010). However, low levels of total protein are also associated with impairment of usual liver functions in sheep diagnosed with pregnancy toxemia (Solouma et al., 2011).

In pregnant ewes, cholesterol levels are reported to be lower from 18 to 50 days while increasing significantly from 100 to 150 days of pregnancy. Similar results were reported by Krajnicakova et al. (1993), Hamadeh et al. (1996), and Nazifi et al. (2002). This decrease in plasma cholesterol levels is compatible with increased energy requirements and negative energy balance (Antunović et al., 2011). It could be attributed to an increase in the absorption of cholesterol by the mammary gland, which

is involved in the synthesis of milk (Nazifi et al., 2002; Piccione et al., 2009). Karadaş. (2008) reported that HDL-cholesterol levels fluctuated throughout pregnancy, tended to fall towards the end of pregnancy, and increased again in the postpartum period. In another study, lower serum HDL-cholesterol levels were obtained in single and twin-bearing ewes, and there was no significant difference between the groups (Akbulut et al., 2021).

In the present study, Plasma urea levels were higher between 50 to 150 days of pregnancy and postpartum than at the first days of gestation. These results are consistent with those reported by El-Sherif and Assad (2001), Antunović et al. (2002), Deghnouche et al. (2011), Ghanem and El-Raof (2012), and Varanis et al. (2021), who reported plasma urea to start increasing in pregnant ewes from the 10th week of pregnancy, reaching a maximum level at parturition.

Serum urea concentration is directly related to dietary protein levels, as well as the protein-energy ratio of the diet (Wittwer et al., 1993). Pregnant ewes have reduced feed consumption in late gestation. This may lead to increased proteolysis of endogenous amino acids to be used as an energy source, occurring in increased urea concentrations (Feijó et al., 2014). The significantly high plasma urea level may be due to increased cortisol levels affecting the catabolism of protein in the body (Silanikove, 2000). Other studies support the hypothesis that changes in blood urea concentration depend on milk synthesis (Dubreuil et al., 2005).

Creatinine comes from the breakdown of creatine, itself synthesized by the liver and stored in the muscles where it plays an important role in energy production. In muscles, approximately 1-2% of free creatine is converted daily to creatinine (Burtis and Ashwood, 1999). The velocity of urinary flow mainly modifies creatinine excretion, and a glomerular filtration rate decreases when physiological values reach higher levels (Ognik et al., 2015). Doornenbal et al. (1988) found that serum creatinine concentration decreased during lactation and increased during post-weaning. At the same time, Solouma et al. (2011) reported that the effect of the breeding period on creatinine concentrations was insignificant. In our study, the values of plasma creatinine in pregnant ewes were lower on days 01-30 of gestation. At the same time, a significant increase starts from day 30 of pregnancy, reaching a higher value at day 100 before decreasing significantly before lambing and during the postpartum period. These variations in plasma creatinine levels during pregnancy and postpartum indicate a protein-deficient metabolism (Piccione et al., 2009) or increased thyroid activity during pregnancy (El-Sherif and Assad, 2001). Changes in plasma creatinine levels were due to muscle catabolism during pregnancy (Fazio et al., 2011). It is well known that different reproductive stages influence ewes‘ levels of various metabolites (Piccione et al., 2009) because of changes in protein and energy demand during these different stages.

Conclusion

It can be concluded that the physiological status of Boujaâd sheep significantly influenced some biochemical parameters during pregnancy and postpartum periods. In pregnant ewes, triglyceride levels increased substantially from 18 to 50 days, compared with non-pregnant ewes whose triglyceride concentrations remained stable from this study‘s start. Consequently, triglycerides may indicate early pregnancy diagnosis in Boujaâd sheep with the primary consideration that the test is carried out on healthy animals, maintained under the same management, and on a short-limited period with a control from the same farm. Therefore, the pregnancy stages significantly influence plasma levels and biochemical parameters. Accordingly, this encourages the need for animal metabolic profile monitoring to determine ewes‘ reproductive and nutritive status. However, more detailed studies on other parameters are required to assess their impact on early pregnancy diagnosis and implement the study in a large group of animals.

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Statements and Declarations

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Author Contributions

All authors contributed to the study's conception and design. Zineb MOUSSAFIR, Anass BEN MOULA, Larbi ALLAI, Abdelati OUAMANI, Boubker NASSER, Khalid RAKIB, Abdelkhalid ESSAMADI, and Bouchra El AMIRI performed material preparation, data collection, and analysis. Zineb Moussafir wrote the first draft of the manuscript, and all authors commented on previous versions. All authors read and approved the final manuscript.

Ethics approval

All the experimental procedures involving animals in this study were conducted according to the Institutional Animal Care Guidelines of the National Institute of Agronomic Research (INRA), Morocco, and approved by the Administration Committee of Experimental Animals, INRA, Morocco (number: 01/CRRAT/2017).

Conflicts of interest

None of the authors have any conflict of interest to declare

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