A plasma protein profile and acute phase proteins in sheep with normal parturition and after cesarean section in cases of dystocia

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Abstract. *The purpose of this study was to define alterations in concentrations of total protein, albumin, globulins, albumin/globulin ratio, as well as fibrinogen and ceruloplasmin (as an acute phase protein) in sheep after normal parturition and in animals with caesarean section. For that, twelve Pleven Blackhead sheep were observed and divided into two groups: controls, which include six females with normal parturition; the second group consisted of six sheep with caesarean surgery. Blood samples were collected into heparinized tubes before parturition and cesarean section (hour 0) and then at hours 6, 24, 48 and on days 4, 8, 10, 12, 14, 18 after labor. During the experimental period, the values of fibrinogen in the sheep with caesarian section were higher than those in the control group. Furthermore, after birth fibrinogen levels gradually increased in both groups of animals. The highest value after normal parturition was observed on day 8, and in the sheep after caesarean section, the highest value was observed on day 10. The ceruloplasmin levels in sheep with normal birth did not change significantly, but within the group of caesarean section, plasma concentrations were markedly increased at 48 hours after the surgery and were maximal on day 10. In normal parturition sheep, the concentration of albumin did not change significantly during the test period, and it was higher than in sheep with caesarian section. The globulin concentrations in animals with caesarian sections were higher than in those with normal labor, with differences statistically significant at day 8 and day 10 after birth. No significant change in the total protein concentration was observed during the whole study period.*

Introduction

The physiological response of the organism to general and local inflammations is connected with an initiation of events leading to a systemic response named acute phase reaction. The changes during this reaction include fever, leukocytosis and quantitative and qualitative modification of total plasma protein present in blood and non-structurally related proteins known as acute phase proteins (APPs) (Cray et al., 2009; Ceciliani et al., 2012; Eckersall, 2019). A plasma protein profile includes albumin and globulins with an expected albumin to globulin ratio of 1:1 in healthy animals (Kaneko, 1980). The inflammatory processes play an important role in the regulation of total plasma protein concentrations with a negative correlation between globulins and albumin and significant changes in the plasma concentrations of acute phase proteins (Eckersall, 1995; Eckersall, 2000; Ceciliani, 2002; Tóthová et al., 2016). An increasing concentration of APPs in blood circulation can be a biomarker for onset and/or development of infectious disease; besides, it has proven to be a very useful indicator for early detection of subclinical inflammation. In addition, a decreasing concentration of APPs is attributed to the recovery stage after treatment of inflammatory disease and can be a powerful tool for monitoring of the treatment (Cray et al., 2009; Eckersall and Bell, 2010). Acute phase proteins have been a focus of many investigations in veterinary medicine and recently have been directed to the veterinary obstetrics and reproduction (Kaya et al., 2016; Costa et al., 2018; Smits et al., 2018; Eckersall, 2019).

In sheep, different APPs (α1-acid glycoprotein, haptoglobin, serum amyloid A and fibrinogen) have shown as biomarkers for the presence of acute or chronic bacterial infection of the respiratory system (Pfeffer and Rogers, 1989) and in ovine caseous lymphadenitis (Eckersall et al., 2007; Bastos et al., 2011). A few researches (Scott et al., 1992; Aziz and Taha, 1997; Georgieva et al., 2011) presented the changes in the haptoglobin concentration in case of dystocia in this animal species. Because the plasma concentration of fibrinogen (Fb) and ceruloplasmin (Cp) increase during the acute inflammation response, those APPs are considered positive (Ceron et al., 2005), whereas plasma proteins whose concentrations decrease during the acute phase response were classified as negative APPs. Also, APPs are classified into major, moderate and minor according to the degree of increase. Ceruloplasmin is considered as a

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minor (< 2-fold increase) APP in ruminants, whereas fibrinogen is a moderate (2–10-fold increase) APP in the same species (Murata et al., 2004).

Fibrinogen, which is produced in the liver, is a β-globulin, considered as an acute phase protein which presents in the plasma of all vertebrates (Ceron et al., 2005). Fibrinogen is included in homeostasis as an integral part of it, providing a substrate for fibrin formation, as well as in tissue repair, providing a matrix for the migration of inflammatory-related cells (Thomas, 2000). Besides, it participates not only in coagulation but also in maintaining pregnancy; therefore, observations on the dynamics of fibrinogen levels are important for safely prenatal and peripartum periods (Teraoka et al., 2017). The main source of Cp is hepatocytes and it can also be synthesized in the mammary gland and places of tissue damage (Szczubiał et al., 2012). However, the information about a plasma protein profile and different acute phase proteins in sheep with a normal and difficult parturition is limited.

The present work aims to determine the changes in a plasma protein profile and certain acute phase proteins in clinical healthy sheep with a normal parturition and puerperal period without reproductive complications as well as in animals after caesarean section.

Materials and Methods

Animals, surgery and blood collection

The study was conducted on twelve Pleven Blackhead sheep, 2–5 years old and weighing 45–60 kg fed and housed under uniform conditions and subjected to the standard immunoprophylactic and antihelminthic regimens.

The control group included six clinical healthy sheep with a normal parturition and puerperal period without reproductive complications or presence of other diseases. All lambs (n = 10; 4 twins and 2 singletons) were housed with the dams in individual pens until the end of the experiment. The second group consisted of 6 females with dystocia presented for veterinary assistance. The reasons for dystocia were uterine torsion (leading to the death of all lambs – 3 twins), oversized fetus (2 live singletons) and ring womb (1 live singleton) in three, two and one cases, respectively. Immediately after the obstetrical examination, a caesarean section was carried out in the animals with dystocia. The anesthesia included intravenous administration of xylazine hydrochloride (0.2 mg/kg, Xylazine 2%, Alfasan International B.V., the Netherlands) and a local infiltration of 20 mL of novocaine (Novocain 1%, Vetprom, Radomir, Bulgaria) subcutaneously into the operative area located in the left flank; postoperative analgesia included intravenous administration of methamizol sodium (5 mL, Analgin 30%, Biovet, Peshtera, Bulgaria) for five days. Among the 9 lambs born from females with dystocia, six were dead. The presence of autolytic changes was indicative of the death occurrence at least 12 to 24 hours before surgery. The uterine involution was monitored and only in the cases of a uterine torsion it was delayed. All sheep were hospitalized after surgery for three weeks.

Individual blood samples were collected from *v. jugularis* prior to 0 hour when it was possible and 4, 24, 48 hours after the part as well as on days 4, 8, 14 and 18. In animals with a normal parturition, the time-point *0 hour* was accepted as the first stage of labor. However, in animals with dystocia, this time-point was delayed and the first blood collection was performed immediately before the surgery. Blood samples were collected in heparinized sterile tubes and were centrifuged immediately (1500 g, 15 minutes, 4°C) for separation of the plasma. Thereafter, plasmas were decanted and stored at –20°C until analysis. All samples were free of hemolysis. The study was performed in accordance with the requirements of Animal Ethics Committee and regulations for human attitude and animal protection.

Plasma protein profile and acute phase proteins assessment

The total serum protein concentration in sheep was determined by the biuret method (Kolb and Kamishnikov, 1982). The serum albumin concentration was determined by a Human test containing bromine-cresol green, SU-ALBU INF 156001F, Gesellschaft für Biochemica, Germany, mixing 10μL plasma with 1 mL of the prepared reagent. After 3 min, the sample was read at λ = 546 nm and the result was calculated using a standard sample at a known concentration (40 g/L). The determination of the globulin concentration was calculated as the difference between the values of total protein and albumin. With the albumin and globulin results, the albumin/globulin ratio (A/G ratio) was calculated.

The concentration of ceruloplasmin was determined by the Ravin method based on the oxidation of p-phenylenediamine (Kolb and Bestujeva, 1982). Since 1970, there have been many modifications to the method, but the PPD oxidase procedure has been widely adapted for routine use in clinical laboratories. From ceruloplasmin and p-phenylendiamine, at pH 5.5, a colored oxidation product was formed and the change in absorption was determined at λ = 530 nm. The plasma fibrinogen concentration was determined by the Podmore nephelometric method with 10% Na_2SO_4 at λ = 570 nm (Todorov, 1972). To 0.25 mL of plasma, 2.5 mL of 10.5% Na_2SO_4 was added against a control sample of 0.25 mL of plasma and 2.5 mL of 0.9% solution of NaCl. The extinction was counted after 3 min at a wavelength of 570 nm, and the result was calculated by multiplying by a coefficient calculated on a standard curve based on various plasma dilutions in which the fibrinogen concentration was determined keldalometrically.

Statistical analysis

Statistical processing of the results obtained in the individual experiments was performed by ANOVA (Statistics for Windows, Stat Soft Ins., USA, 1993). The statistical significance of intra- and intergroup differences was determined by the post hoc procedure LSD test (Stat Soft Ins., USA, 1993). The level of statistical significance of the differences was at $P < 0.05$.

Results

The obtained results that relieve the effect of normal parturition and caesarean section on concentration of fibrinogen and ceruloplasmin are shown in Figures 1, 2, 3, 4, 5 and 6. Fig. 1 presents data on the amount of fibrinogen (g/L) in the blood plasma of sheep born with caesarean section and normal parturition sheep. The concentration of Fb in the blood of sheep before normal parturition was 1.98 ± 0.25 g/L (0 h), and in the plasma of operated sheep, it was 2.22 ± 0.17 g L. After birth, fibrinogen levels gradually increased in both groups of animals, with values significantly higher ($P < 0.05$; $P < 0.01$) in both groups at 48 hours compared with the pre-natal period. The highest value after normal parturition (4.63 ± 0.25 g/L) was observed on day 8, and in the sheep after caesarean section, it was observed on day 10^h (7.11 ± 1.31 g/L, $P < 0.001$). Significantly higher values after normal parturition in sheep persisted until day 12, and in those with caesarian section until day 14. At the end of the study period (day 18), fibrinogen concentrations in both groups were approaching baseline levels. Throughout the experimental period, the levels of fibrinogen in the sheep with caesarian section were higher than those in the control group, with differences between day 10 ($P < 0.001$), day 12 ($P < 0.01$) and day 14 ($P < 0.01$).

Results about the concentration of ceruloplasmin (mg/L) in the blood plasma of sheep with caesarean section and sheep with normal parturition are presented in Fig. 2. Throughout the experiment, the ceruloplasmin in sheep with normal birth did not change significantly. It varied from 170.06 ± 5.79 mg/L before birth and 164.45 ± 3.09 mg/L on day 18 after birth. There was a non-significant increase on day 10 after birth (194 ± 3.09 mg/L).

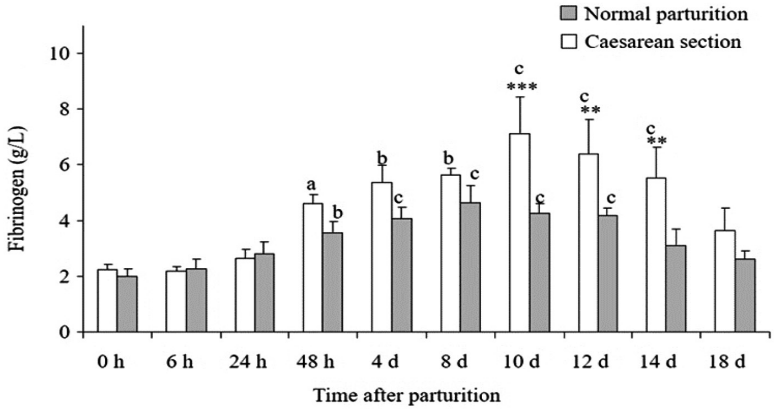

Fig. 1. Fibrinogen concentrations in blood plasma of sheep with normal parturition (n = 6) and animals with dystocia submitted to caesarean section (n = 6).
Results are expressed as means ± standard errors of the means.
Significance of the differences between the groups: ** $P < 0.01$; *** $P < 0.001$
Significance of the differences within the groups: a ($P < 0.05$); b ($P < 0.01$); c ($P < 0.001$).

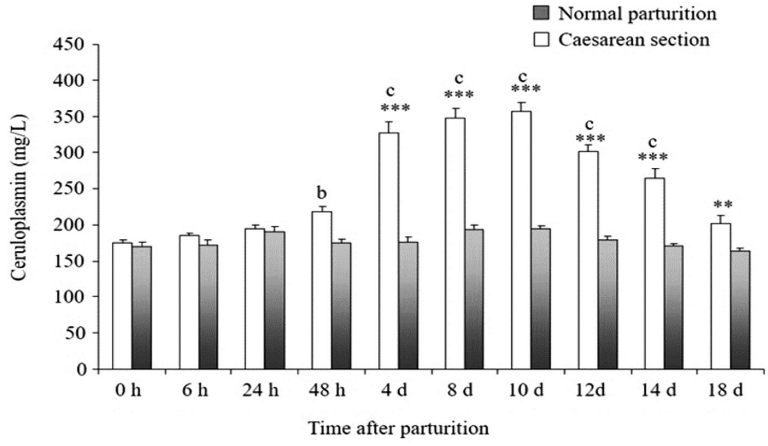

Fig. 2. Ceruloplasmin concentrations in blood plasma of sheep with normal parturition (n = 6) and animals with dystocia submitted to caesarean section (n = 6).
Results are expressed as means ± standard errors of the means.
Significance of the differences between the groups: ** $P < 0.01$; *** $P < 0.001$.
Significance of the differences within the groups: b ($P < 0.01$); c ($P < 0.001$). h – hours; d – days.

In cesarean sectioned sheep, a significant increase in the concentration of ceruloplasmin was observed since day 4 (326.5 ± 15.87 mg/L) until day 18 (201.83 ± 10.8 mg/L) ($P < 0.001$). It was highest on day 10 after the intervention – 357.04 ± 12.44 mg/L.

Within the group of Caesarean section, the significance of differences was reported at 48 hours after surgery ($P < 0.01$), and then increased, reaching maximum values since days 4 to 14 ($P < 0.001$).

Data in Fig. 3 show the concentration of albumin (g/L) in the blood plasma of sheep with cesarean section and normal parturition sheep. The level of albumin in the blood of sheep before normal parturition was 30.7 ± 2.3 g/L (0 h), and in the plasma of operated sheep, it was 33.44 ± 2.79 g/L. After the birth, its level decreased in the operated group, with significant differences ($P < 0.05$) compared with baseline values on days 8 and 10. The lowest value was reported on day 10 in the cesarean group – 26.91 ± 1.99 g/L. At the end of the study period (day 18), the level of albumin in the test group returned to its original values – 33.58 ± 3.12 g/L. In normal parturition sheep, the concentration of albumin did not change significantly during the test and ranged from 30.7 ± 2.3 g/L (0 h) to 29.35 ± 2.47 g/L (day 18).

The information in Fig. 4 represents the concentration of globulins in the blood plasma of sheep with cesarean section and sheep with normal parturition. No significant difference was observed in the level of globulin in the plasma of normal-parturiated sheep. Before parturition, it was 32.36 ± 2.32 g/L (0 h), and at the end of the study period, it was 32.33 ± 2.33 g/L (day 18).

In the plasma of operated sheep at the beginning of the period, the level of globulins was 35.53 ± 2.12 g/L. After surgery, its level increased significantly on day 8 to 44.1 ± 3.33 g/L ($P < 0.05$), after which the concentration of globulins gradually decreased at the end of the study period (day 18). In the cesarean section group, it was close to its original values – 35.18 ± 2.02 g/L. Throughout the experimental period, the globulin concentrations in sheep with caesarian sections were higher than those with normal parturition, with differences statistically significant ($P < 0.01$) at day 8 and day 10 after the birth.

As shown in Figure 5, the A/G ratio for operated sheep and sheep that gave birth normally up to 24 hours of the study period was almost the same, i.e., about 0.95 ± 0.03 (controls). At 24 hours after the birth in operated animals, it began to decrease, with differences from baseline values statistically significant at 48 hours ($P < 0.05$), day 4 ($P < 0.001$), day 8 ($P < 0.001$), day 10 ($P < 0.001$) and day 12 ($P < 0.01$). The lowest value was reported on day 8 – 0.61 ± 0.03. This means that globulin synthesis is significantly increased at the expense of albumin synthesis. After day 14, the A/G ratio values returned to their original levels again – 0.908 ± 0.04 (for the sheep with normal parturition) and 0.95 ± 0.05 for cesarean operated sheep (Fig. 5). In the postpartum period, A/G ratios in sheep with caesarean section were lower than in normal parturition sheep, with differences between groups statistically demonstrated at day 4 ($P < 0.001$), day 8 ($P < 0.001$), day 10 ($P < 0.001$) and day 12 ($P < 0.01$).

As reported in Fig. 6, no significant change in total protein concentration was observed during the whole study period, both in control animals (normal birth) and in the operated ones. At the beginning of the period, it was 68.97 ± 4.72 g/L and 63.07 ± 4.48 g/L, respectively (0 h). On day 18 after birth, the total protein content was 68.76 ± 4.8 g/L (operated) and 60.73 ± 5.14 g/L (controls). It should be noted that

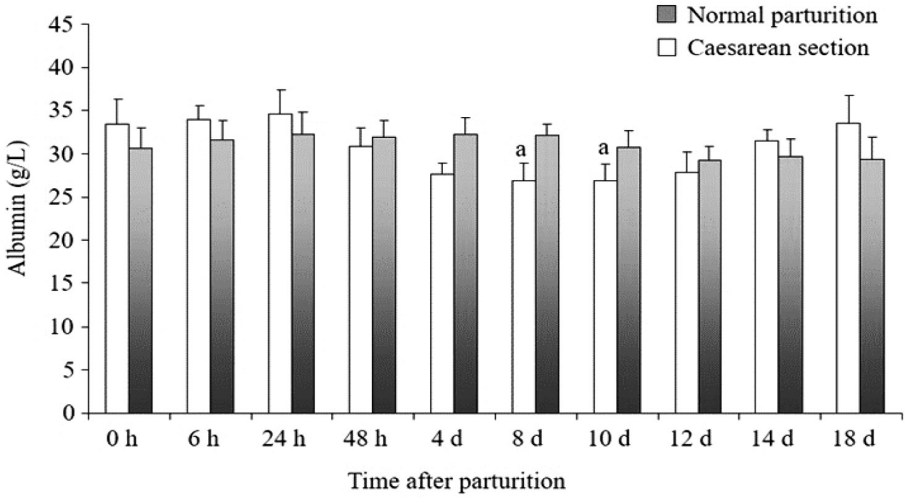

Fig. 3. Albumin concentrations in blood plasma of sheep with normal parturition (n = 6) and animals with dystocia submitted to caesarean section (n = 6).
Results are expressed as means ± standard errors of the means.
Significance of the differences within the group with caesarean section: $^{a}P < 0.05$; h – hours; d – days.

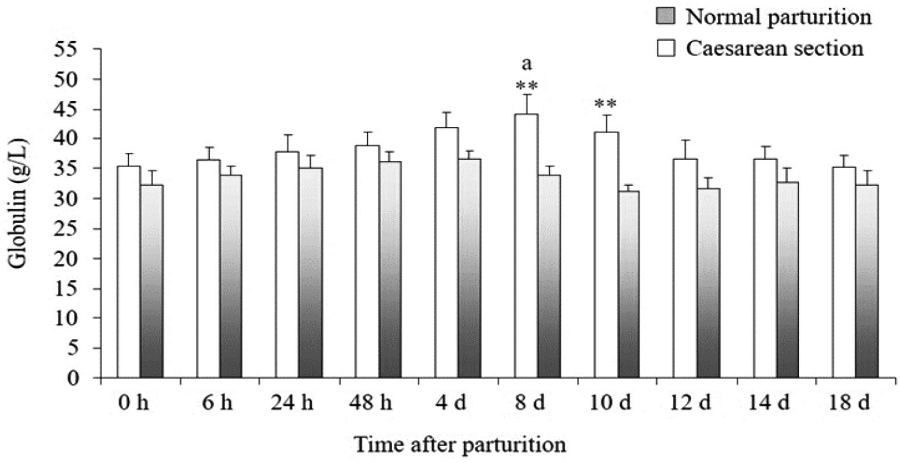

Fig. 4. Globulin concentrations in blood plasma of sheep with normal parturition (n = 6) and animals with dystocia submitted to caesarean section (n = 6).

Results are expressed as means ± standard errors of the means.
Significance of the differences between the groups: $^{**}P < 0.01$.
Significance of the differences within the group with caesarean section: $^{a}P < 0.05$; h – hours; d – days.

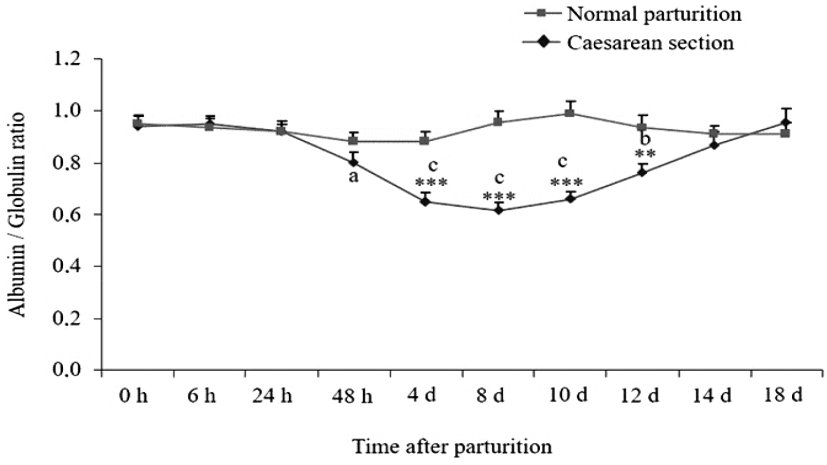

Fig. 5. Albumin/globulin ratio in blood plasma of sheep with normal parturition (n = 6) and animals with dystocia submitted to caesarean section (n = 6).

Results are expressed as means ± standard errors of the means.
Significance of the differences between the groups: $^{**}P < 0.01$; $^{***}P < 0.001$.
Significance of the differences within the groups: $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$; h – hours; d – days.

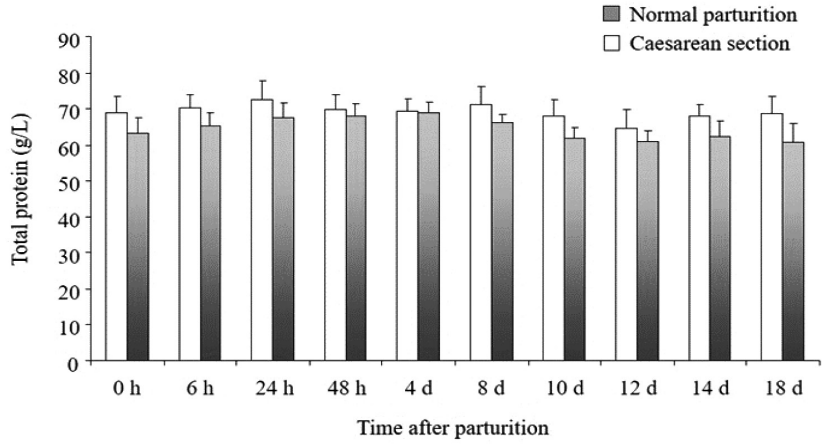

Fig. 6. Total protein concentrations in blood plasma of sheep with normal parturition (n = 6) and animals with dystocia submitted to caesarean section (n = 6).

Results are expressed as means ± standard errors of the means.
No significance of the differences between or within the groups; h – hours; d – days.

throughout the study, total protein levels in sheep born with caesarean sections were higher than those in normal sheep, but statistically the differences were not significant.

Discussion and conclusions

In this study, we describe raised concentrations of certain APPs in sheep with normal parturition and animals with dystocia submitted to caesarean section. The acute phase proteins are substances synthesized during an acute phase response (APR) which is caused by inflammation, infection, trauma or tissue damage (Ceron et al., 2005; Petersen et al., 2004). It is stimulated by the release of cytokines (IL-1, IL-6, TNF-α) from macrophages and monocytes at the site of inflammatory lesions or infections. The synthesis and role of APPs may differ depending on the animal species. However, Murata et al. (2004) announced that APP may be applied to evaluate not only inflammatory conditions, but also some non-inflammatory processes such as parturition, pregnancy, stress, metabolic diseases. The changes in concentrations of APPs caused by a different stimulus are fast, with maximum levels about one day after initiation and before returning to their baselines within a week (Meling et al., 2012). It should be noted that most APPs have a half-life of only 24 to 48 hours (Cray et al., 2009).

Physiological and metabolic changes around parturition can be regarded as an event which causes potential stress on livestock, resulting in activation of the APR and subsequent increase in concentrations of positive APPs (Tóthová et al., 2014). The acute phase response in ruminants is radically different from other animals and the APR studies on sheep are not as large as in cows, but it is thought to be similar. Pfeffer and Rogers (1989) found that, following the development of pneumonia, sheep observed elevations in plasma ceruloplasmin and fibrinogen, decreased erythrocytes, and increased neutrophils by 250%, 400%, 80%, and 200%, respectively. The authors recommend measuring the level of APP instead of the number of circulating neutrophils. On the other hand, Ulutas and Ozpinar (2006) found that, in annual lambs infected with *Pasteurella haemolytica*, serum levels of APP like haptoglobin, ceruloplasmin, and fibrinogen increased during infection. Razavi et al. (2011) found that serum amyloid A (SAA) greater than 57.15 μg/mL, haptoglobin greater than 0.42 g/L, ceruloplasmin greater than 0.27 g/L and fibrinogen greater than 3.91 g/L were appropriate indicators for an inflammatory process in sheep infected with *T. lestoquardi*. As parasitemia levels increased (< 2%, 2–4% and > 4%), levels of haptoglobin, ceruloplasmin, and fibrinogen increased.

The dynamics of changes in the concentration of fibrinogen in sheep with normal parturition are similar to those in sectioned animals. In all animals, the levels were increased significantly ($P < 0.05$) at 48 hours, returning to baselines at 14 days in the group with normal birth. At the same time, in the group with caesarean section, they were higher compared with initially registered ($P < 0.001$).

Ceruloplasmin, which belongs to the group of minor APPs who react with a slight increase to the stimulus, has also increased since 48 hours, but only in sheep with caesarean section, whereas in normal birth animal, the dynamics remain almost unchanged throughout the study period. In both groups of sheep, the sectioning operation caused a maximal increase in Fb and Cp values on day 10 ($P < 0.001$). Measurements of APPs can be used to evaluate the innate immune system response to pathological injuries and may even be possible to use them as markers to assess the overall health of the herd in farm animals (Meling et al., 2012).

The protein profile has been applied in studies on sheep to identify any pathological conditions during stressful conditions such as parturition and cesarean surgery. It should also be taken into account that albumin is considered as a negative acute-phase protein that responds to stress by lowering levels. In this study, changes in blood protein profiles were observed in sheep with normal parturition and animals with dystocia submitted to caesarean section. According to Gruys et al., (2005) albumin in all animals and humans, birds and fish related to the group of "negative" acute phase proteins, which means that its concentration decreased after inflammation or infection. Hypoalbuminemia is very common in many diseases and results from a disorder in liver synthesis, reduced absorption of amino acids or increased catabolism linked to the turn of amino acids for the synthesis of other proteins (like positive APPs) in liver, or a combination of these factors (Georgieva et al., 2011). Globulins are a group which contain some fractions-α_1, α_2, β and γ. The results obtained show that labor is accompanied with a moderate, but significantly increasing concentration of globulins, decreasing albumin and A/G ratio as well as increasing APPs – haptoglobin, ceruloplasmin and fibrinogen (Georgieva, 2013) and proves that there is an inflammatory process that precedes the clinical signs of difficult birth and/or fetal death. However, in our work, we demonstrated that caesarean section in sheep can be considered as a cause of change in the protein profile. Decreasing albumin values in the operated group in the present study confirm that albumin may be considered as a negative APP in sheep.

The total protein is an indicator with relatively constant values. In different groups of animals, it ranges from 39 g/L to 85 g/L according to some authors, and from 50 g/L to 75 g/L according to others. No significant change in the total protein concentration was observed during the whole study period, both in animals with normal birth and in the operated ones. Likewise, in sheep with caesarean

sections, values of total protein were higher, but not statistically, than those in sheep with normal parturition. This might be explained by producing a large number of APPs by the liver which are present at very low concentrations in a normal state, but during inflammation, trauma or surgical invention, most of them which are related to the group of positive APPs (Jain et al., 2011) increased several times, and could elevate the concentration of total protein in caesarean section sheep, because during operation restricted inflammation is developed. Gürgöze et al. (2009) investigated changes in some biochemical parameters in Awassi ewes throughout pregnancy and postpartum period. Similarly to our results, they reported that during the whole studied period total protein and albumin concentrations were in the reference range for sheep.

We suggest that changes in studied plasma protein parameters must be taken into account when considering the biochemical status of sheep in the days after birth.

In the present study, the result shows the presence of variation in the plasma protein profile and investigated acute phase proteins. A significantly ($P < 0.05$) increasing concentration of globulins, a decreasing concentration of albumin and albumin/globulin ratio were observed in plasma of sheep with dystocia submitted to caesarean section compared with animals with normal parturition. Similarly, it was examined that certain acute phase proteins such as albumin (as negative APP), and fibrinogen and ceruloplasmin (as positive APPs) can be useful as prognostic indicators for monitoring the postpartum period in sheep.

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