

Original Article

Neutrophil phagocytosis and respiratory burst activity of dairy cows during the transition period and early lactation

Marina Žekić-Stošić¹, Zdenko Kanački², Dragica Stojanović², Dejan Bugarski¹, Miodrag Lazarević³, Aleksandar Milovanović¹, Aleksandar Masic^{4,5}

¹ Scientific Veterinary Institute "Novi Sad", Novi Sad, Serbia

² Department of Veterinary Medicine, Faculty of Agriculture, University of Novi Sad, Novi Sad, Serbia

³ Faculty of Veterinary Medicine, University of Belgrade, Belgrade, Serbia

⁴ NovaVive Inc, , Ontario, Napanee, Canada

⁵ Faculty of Ecological Agriculture, EDUCONS University, Sremska Kamenica, Serbia

Abstract

Introduction: Hormonal and metabolic changes, as well as energy imbalance, can affect health, production and reproductive performance of dairy cows. In the present study, we evaluated phagocytosis and respiratory burst neutrophil activity during the transition period and early lactation and compared it with biochemical and hematological parameters in dairy cows.

Methodology: Simmental cows (n = 21) were enrolled in the study. Whole blood samples were collected weekly from 3 weeks pre-calving until 6 weeks post calving. Basic metabolic and blood parameters were assessed by routine laboratory analyses, while neutrophil functions were analyzed by commercial test kits.

Results: Optimal neutrophil response was observed pre and post calving. The highest value was recorded in the 6th week after calving (89.54 ± 7.61%) and being significantly higher (p < 0.01) as compared to values recorded at two and one week before and one week after calving. The percentage of activated neutrophils was high during the entire study period: from 70.80 ± 5.22% at the beginning of the study to 89.54 ± 7.61% at the end of the study. During the study period, production of Reactive Oxidative Species by neutrophils was positively correlated with β-hydroxybutyrate and non-esterified fatty acids values (0.454** and 0.423**, respectively) and calcium levels (0.164* and 0.212**, respectively).

Conclusions: The most prominent changes in all parameters had no influence on phagocytic and respiratory burst activity of neutrophils. Neutrophil function is preserved at the optimal level during the transition period and early lactation in Simmental cows.

Key words: dairy cows; neutrophil; phagocytosis; respiratory burst.

J Infect Dev Ctries 2018; 12(10):894-903. doi:10.3855/jidc.10767

(Received 13 August 2018 – Accepted 29 September 2018)

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Introduction

The peripartum period (3 weeks before and 3 weeks after calving) is characterized by significant endocrine and metabolic changes in dairy cows. The transition from the gestation to lactation is accompanied by a complex series of hormonal changes and it is the most challenging period for the dairy cow [1]. With the onset of lactation, there is an increased energy and nutritional demand, which requires excessive metabolic adaptations. The peripartum period in dairy cows is characterized by a higher incidence of metabolic disorders, infectious diseases, and by the activation of homeostatic and homeorhetic mechanisms [2]. At least five critical points during the transition period were identified by few groups of authors [3]: a) reduction of immune competence, b) negative energy balance (NEB), resulting in mobilization of the adipose and

muscle tissue, c) hypocalcemia, as a consequence of the delayed availability of calcium in the blood for the sudden and significant demand of the mammary gland for milk synthesis, d) an overt systemic inflammatory response around the time of calving, which commonly occurs immediately after calving, often in the absence of visible signs of microbial infections or other pathological conditions, e) oxidative stress, due to the unbalanced availability of antioxidants caused by events that increase overproduction of potent pro-oxidant molecules.

For a cow to transit from late pregnancy into lactation successfully, she needs to expertly coordinate metabolism in multiple tissues to ultimately provide sufficient glucose to support productive needs. Periparturient diseases result from a complex interrelationship between the cow's ability to manage

metabolic adaptations during transition [4]. The transition period is characterized by social regroupings, pen-moves, cow-calf separation, and for primiparous animals, exposure to novel environments such as the milking parlor [5]. The end of gestation and early lactation in high-yielding dairy cows is mostly marked by metabolic stress which could lead to immunosuppression and consequent infection [6,7]. Neutrophils are actively involved in the ingestion and intracellular killing of invading microorganisms mainly via phagocytosis and intracellular reactive oxygen species (ROS) production [8,9]. The reason for decreased function of polymorphonuclear leukocytes (PMNL) during the periparturient period has been attributed to increased nutritional demands for fetal growth and colostrum production at the end of pregnancy [10]. Most dairy cows experience a negative energy balance around calving period, which can contribute to the impaired function of the immune system. The main source of plasma fatty acids throughout the transition period is the lipolysis of adipose tissue depots. During this period, plasma fatty acids serve as a source of calories, thus mitigating negative energy balance, a consequence of elevated milk synthesis and limited dry matter intake [11]. Elevated non-esterified fatty acids (NEFA) and β -hydroxybutyrate (BHB) levels are consequences of such intensive lipolysis. Levels of calcium, NEFA and BHB are the main endogenous factors with a strong influence on the functional activity of neutrophil granulocytes in the transition period [12]. During the transition period, in addition to negative energy balance (NEB), serum glucose concentration can decrease. Since serum glucose is an important “fuel” for leukocytes during the period of negative energy balance in dairy cows, there is a change in the function of neutrophil granulocytes including reduced killing capacity [13,14]. Change in urea concentration may also affect functional activity of neutrophil granulocytes [15–17] as well as changes in glucocorticoids levels [7]. All these changes can cause impaired functional activity of neutrophils, which are the first line of defense against microbes. It is evident that there is a considerable level of metabolic and hormonal activity during this period, but it still remains unclear which of all of these factors contribute the most to immunosuppression, because neither factor is constant throughout the transition period. For example, glucocorticosteroids are elevated for approximately 24 hours around parturition and immunosuppression has been observed during the first three weeks after parturition and therefore, changes in glucocorticoids cannot be a major contributor to the

immunosuppression observed during the transition period [6].

The ability of cows to resist the disease developing during the periparturient period is related to the efficiency of the immune system. Therefore, the objectives of this study were: to estimate (1) phagocytic and respiratory burst activity of neutrophils, (2) biochemical and hematological parameters related to different peripartum periods (weeks) and (3) to establish correlation of phagocytic and oxidative burst activity of neutrophils with biochemical and hematological parameters during transition period.

Methodology

Experimental design

This study was conducted on twenty-one Simmental cows. Cows were randomly selected from a farm with 120 milking cows, located in the northern part of the Republic of Serbia, Subotica municipality. At the beginning of the study, selected cows were clinically healthy and in late gestation phase (four weeks prior to expected calving date).

Of all cows enrolled in the study, 8 were primiparous and 13 were multiparous (parity ranged from 3 to 7, average 3.6). The average milk production in the studied group was 28.5L per day and cows were milked twice a day. All blood collections were performed once a week throughout seven weeks, beginning at 3 weeks prior to calving until 3 weeks post calving. A final group of samples was obtained at 6 weeks post calving. Body condition scoring (BCS) was estimated according to a 1 to 5 scale [18]. Cows were fed total mixed ration (TMR) based on alfalfa hay, corn and rye silage and concentrated meals for high-yielding cows. Additional vitamin and mineral supplements were offered. Easylin® (VALOREX, La Messayais, Combourtillé, France), as a source of omega-3 fatty acids has been supplemented during the entire study period. TMR was approximately 20% above required energy intake for the average recorded lactation values, allowing a surplus of nutritive requirement for rapid recovery after freshening.

Sample processing and analysis

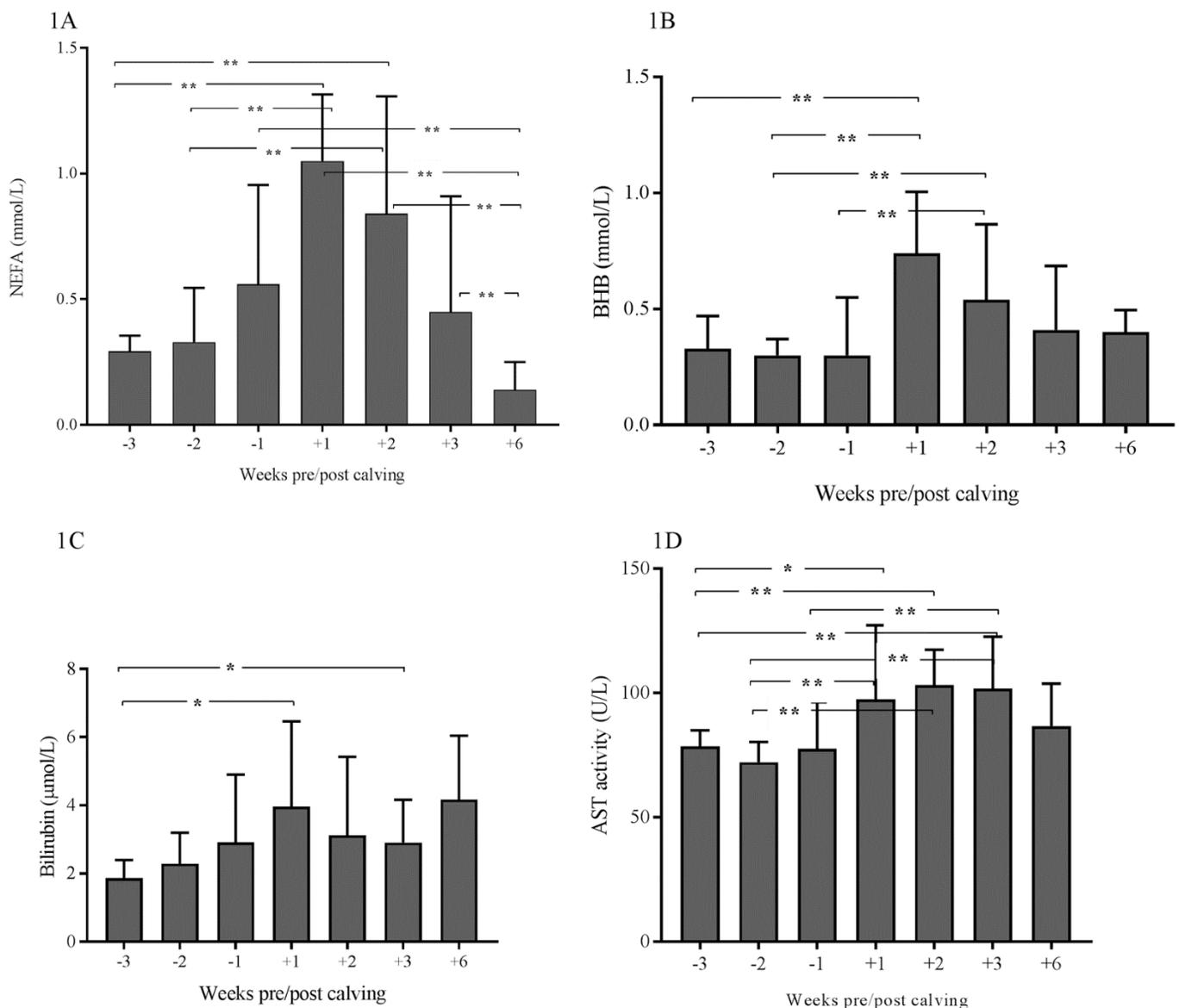
Whole blood samples were collected by venipuncture (*v. coccigea*) in the sterile heparinized tubes (BD Vacutainer®, Plymouth, Great Britain) and kept on ice until delivery to the laboratory for PMNL phagocytosis and respiratory burst test. Meanwhile, non-heparinized tubes (BD Vacutainer® CAT, Plymouth, Great Britain) were kept in cold box and

serum was harvested after centrifugation at 1000×g for 10 minutes.

Biochemical parameters were analyzed in order to estimate the cow’s health status prior to, and after calving as well as during early lactation period. Serum samples were analyzed for the following biochemical parameters: total protein, globulin, albumin, urea, total bilirubin (TB), glucose, non-esterified fatty acids (NEFA), β-hydroxybutyrate (BHB), aspartate transaminase activity (AST), calcium (Ca), phosphorus (P) and magnesium (Mg). Biochemical parameters in the sera were determined by colorimetric reaction, measured by spectrophotometer (Semi-auto

biochemical analyzer Rayto, RT-1904C, Rayto Life and Analytical Sciences Co., Ltd., Shenzhen, China). The solutions and reagents were used according to manufacturer’s instructions (Biosystems, Barcelona, Spain). In addition, whole blood samples were analyzed for hematology parameters: white blood cells count (WBC), neutrophil count, lymphocyte count, lymphocytes (%), monocytes (%), neutrophil (%), red blood cell count (RBC), hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC). The hematological parameters were measured using an automatic

Figure 1. Values of some biochemical parameters (median ± IQR) in sera of dairy cows; A. NEFA concentration (mmol/L); B. BHB concentration (mmol/L) C. Bilirubin concentration (µmol/L); D. AST activity (U/L); *significant differences ($p < 0.05$), **significant differences ($p < 0.01$).



hematological analyzer (Abacus Junior Vet, Diatron MI PLC, Budapest Hungary). The biochemical and hematology results were compared to the reference values [19].

Phagotest and Phagoburst

Commercial kits Phagotest® and Phagoburst® tests (Glycotop, Berlin, Germany; Cat. No 341060 and 341058) were used for quantitative and qualitative evaluation of phagocytosis and oxidative burst activity of neutrophils, originating from the whole heparinized blood. Blood samples were analyzed within 4 hours post collection by Flow-Automated-Cell Sorting (FACS) cytometry EasyCyte (Guava Technologies, Hayward, California, USA), according to the manufacturer’s instructions. The strength of the phagocytosis and oxidative burst activity of neutrophils were presented as an index. The index was calculated by multiplying the percentage of positive cells in reactions and their mean fluorescence intensity (MFI), using the formula % positive cells × MFI/100, as described by [20].

Statistical analysis

Statistical analysis of all collected data was performed using the Statistical software GraphPad Prism version 5.0 (Graph Pad Software, San Diego, California, USA) and MS Excel (Microsoft Corp.). All data (biochemical parameters, hematological

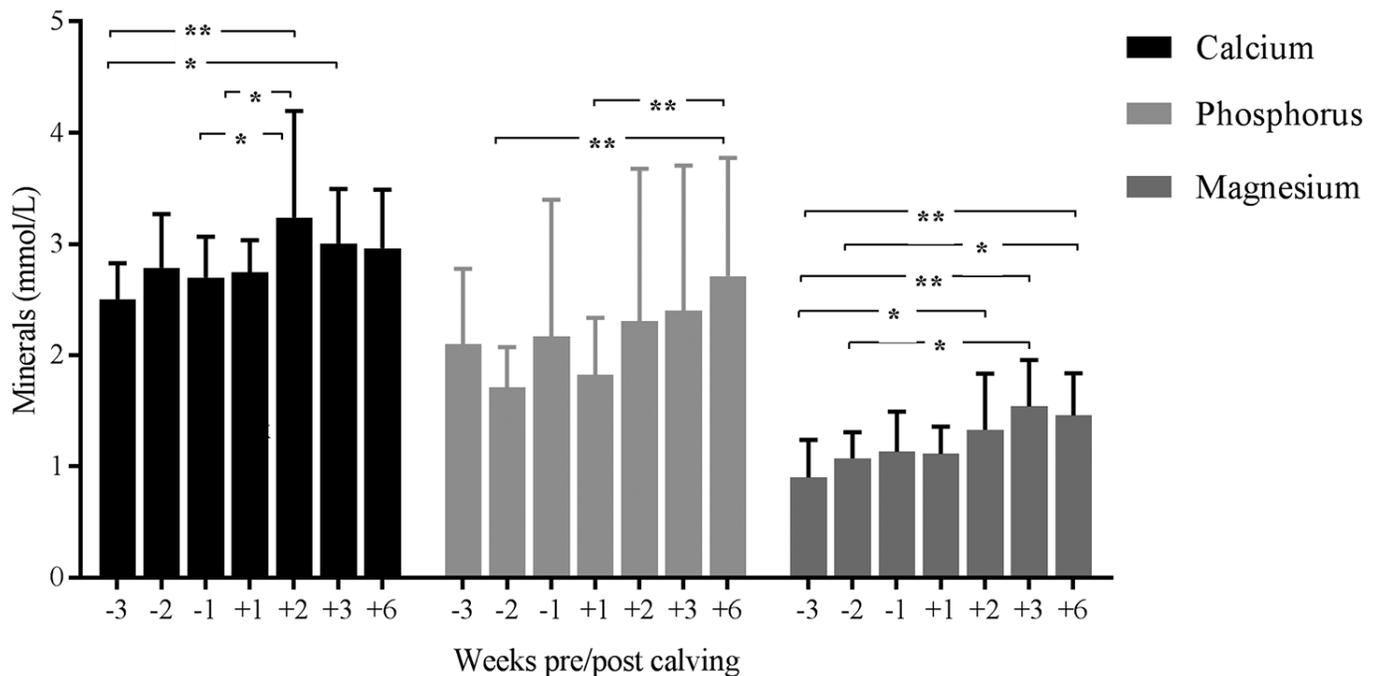
parameters and indicators of phagocytosis and oxidative burst activity of neutrophils) were subjected to statistical analysis using one-way ANOVA, followed by Kruskal-Wallis multiple comparison test. Correlations between variables were also analyzed using the Pearson correlation coefficient.

Results

Serum biochemical parameters analysis

Most of the changes in biochemical parameter values occurred around parturition. NEFA concentrations were rising through the entire prepartum period and reached maximal levels at weeks 1 and 2 post parturition (1.05± 0.50 mmol/L and 0.81 ± 0.67 mmol/L, respectively). BHB concentrations were within physiological range during the study period and reached the highest levels at first week after parturition (0.74 ± 0.43 mmol/L). Significant differences were observed in NEFA and BHB levels, as well as in the samples taken one and two weeks after calving, as compared to other periods of sampling (Figures 1A and Figure 1B). The total bilirubin level (Figure 1C) and AST activity (Figure 1D) increased in the first week after calving. Statistically significant differences between pre – and postpartum values of these two parameters were observed and presented in Figure 1. All minerals levels were within physiological ranges before calving. Ca levels were above the physiological values after calving, while phosphorus levels after

Figure 2. Mean values (±SD) of calcium, phosphorus and magnesium concentrations in sera of dairy cows (mmol/L). *significant differences (p < 0.05), **significant differences (p < 0.01).



calving were below physiological values at first and second week after parturition (1.66 ± 0.59 mmol/L and 1.74 ± 0.77 mmol/L, respectively). Statistically significant differences between mean values of minerals were observed in different periods as shown in Figure 2. The majority of other biochemical parameter values (total protein level, albumin, bilirubin and glucose levels, urea concentration and AST activity) were within the physiological range for cows.

Total protein, albumins, globulins levels and urea concentration decreased after calving, as well as the glucose concentration, yet without reaching significant values (Table 1-S). Minimal loss of body condition score was observed at sixth week of lactation (3.35 ± 0.53) as compared to BCS at third weeks before parturition (3.65 ± 0.28).

Hematological parameters values

Hematological parameters were determined at the same sampling points as the values for biochemical parameters (Table 2-S). Significant changes occurred in Hb, Hct, MCV and MCHC levels. Hb and Hct values decreased in the second week after calving (Figure 3A-B). The highest MCV value was observed in the first week after calving (51.24 ± 4.35 fL; Figure 3C). MCHC value at the end of study (week 6) was significantly higher than the values recorded at all other sampling periods (Figure 3D). A shift of white blood cells count, total neutrophil count, total lymphocyte count and percent of lymphocytes occurred in the week preceding parturition but the changes were within the physiological range reported for transition cows and thus considered not significant. Most of hematological parameters were within the physiological range for

Figure 3. Values of some hematology parameters (median± IQR) in whole blood samples of dairy cows (mean± SD); A. Hemoglobin concentration (g/L); B. Hematocrit values (L/L); C. MCV values (fL); D. MCHC values (g/L); *significant differences ($p < 0.05$), **significant differences ($p < 0.01$).

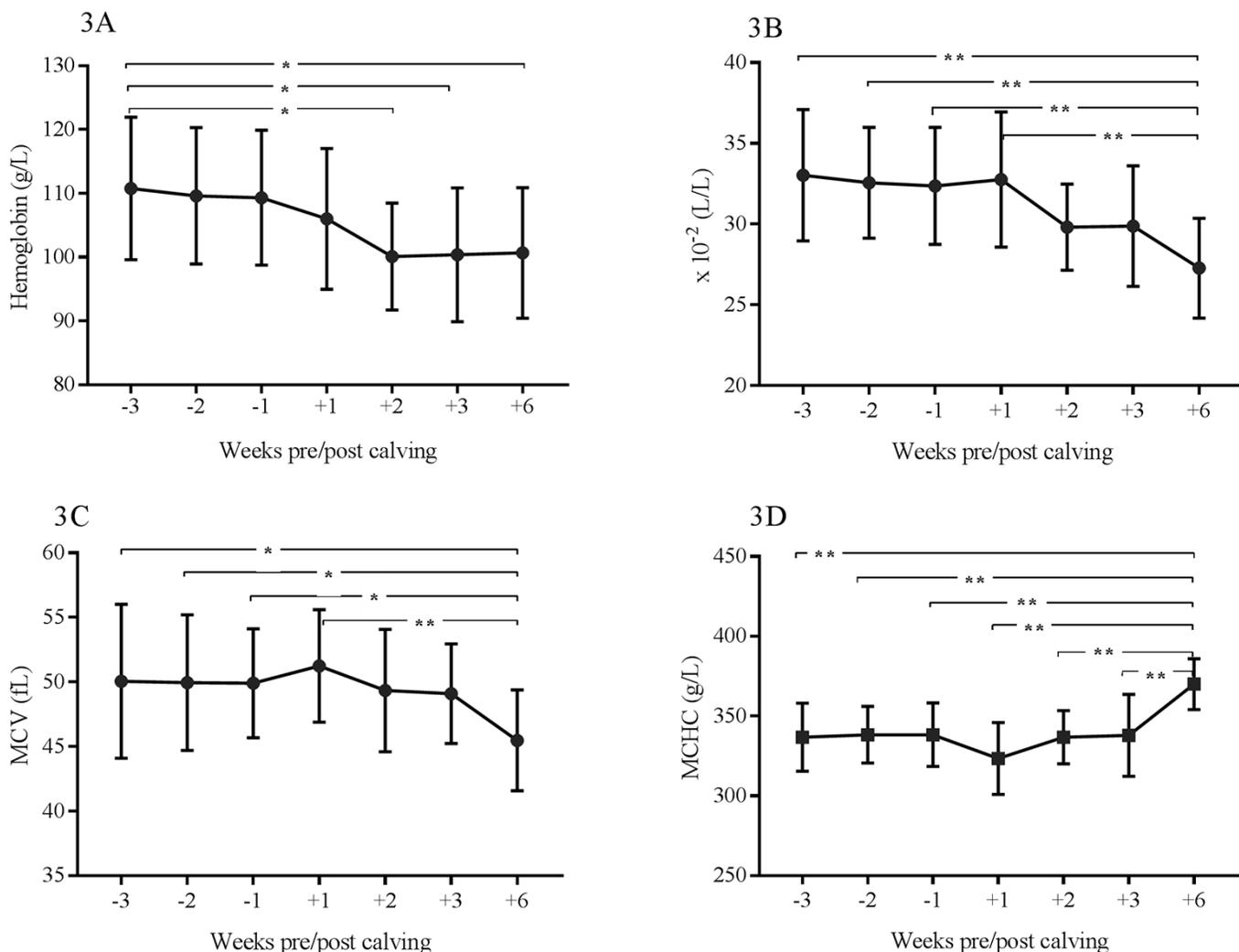
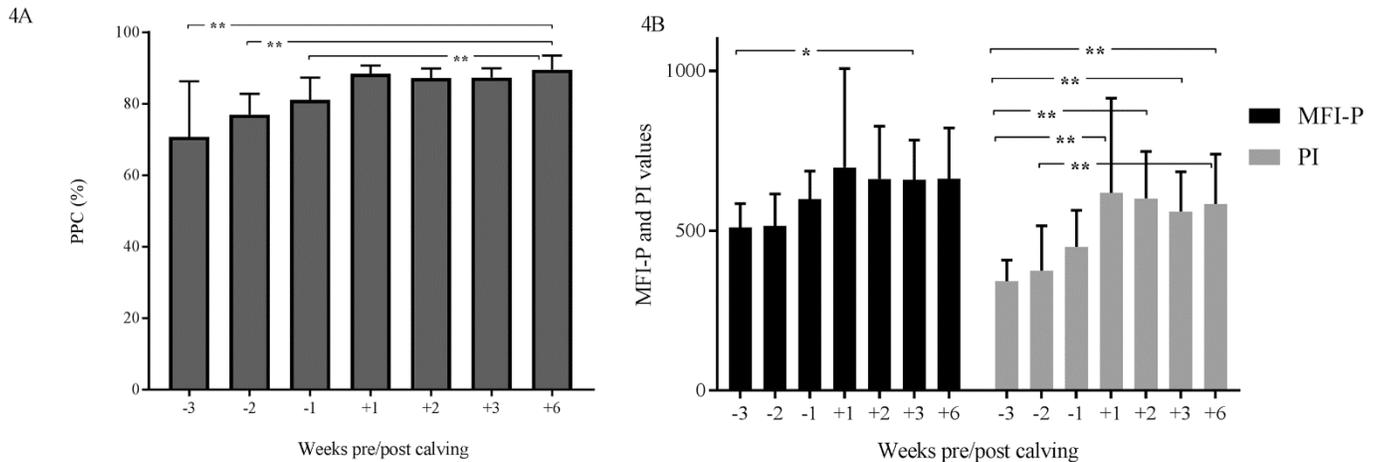


Figure 4. A. Percentage of cells positive for phagocytosis (PPC%) and B. Mean fluorescence intensity (MFI) and index of phagocytosis (PI) following stimulation of neutrophils with fluorescein-isothiocyanate - labeled *Escherichia coli* (mean± SD); *significant differences ($p < 0.05$), **significant differences ($p < 0.01$).



cows. During the whole study period, the percentage of neutrophils was above physiological values, with the highest value one week prior to calving, but without significance also.

Phagocytic activity of neutrophils

Phagocytic activity of neutrophils intensified continuously until the sixth weeks after calving (Figure 4). The mean value for the percentage of activated phagocytic cells was generally high during the entire study period ($70.80 \pm 20.93\%$) as shown in Figure 4A. The proportion of neutrophils that exerted phagocytosis in the sixth week after calving ($89.54 \pm 7.61\%$) was significantly higher than the average values at weeks 2 and 1 before calving and week 1 after calving ($p < 0.01$).

The greatest phagocytosis intensity (expressed as MFI - mean fluorescence intensity) was observed in the first week after calving (Figure 4B). Following that, MFI remained elevated until the end of the study ($p <$

0.05). Phagocytic index (PI) significantly increased ($p < 0.01$) immediately after calving and remained elevated as lactation progressed until week six (Figure 4B).

Correlation of phagocytosis and recorded values for blood parameters and metabolic profile parameters

After summarizing the parameters from all periods, it was established that none of the examined ones had negative influence on phagocytosis. The analysis of the values of metabolic parameters revealed several statistically positive correlations for phagocytosis traits during the entire transition period and early lactation (three weeks before to six weeks postpartum). During the period of observation, concentrations of albumin, Mg^{2+} , Ca^{2+} , BHB and NEFA showed positive correlation with most phagocytosis traits, particularly with MFI and PI. Only correlation coefficients with significant differences are presented in Table 1.

Figure 5. Oxidative burst activity of neutrophils (median± IQR); A. Percentage of oxidative burst positive cells (OPC%); B. Mean fluorescence intensity (MFI) and oxidative burst index (OBI) following stimulation with phorbol 12-myristate 13-acetate (PMA).

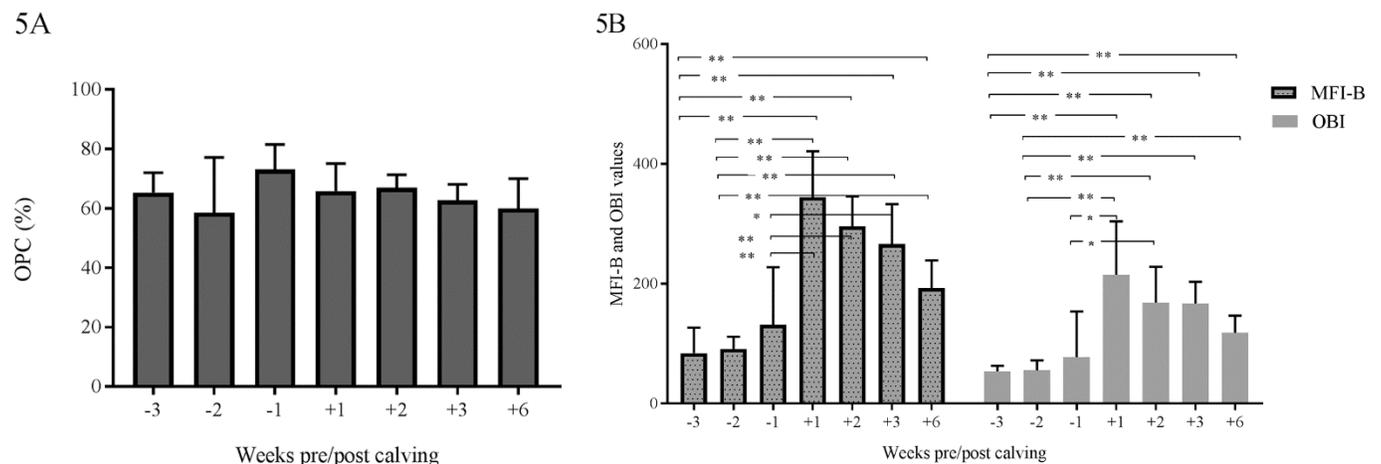


Table 1. Correlation of phagocytosis and metabolic profile parameters before and after calving (total of 147 analyses) based on Pearson correlation coefficients.

Neutrophil activity	Peripartum period and early lactation (-3 to +6 weeks)	
	Positive (+) correlation	Negative (-) correlation
Phagocytosis		
% phagocytic positive cells	Alb (0.179); Mg (0.284**)	-
MFI ¹	Ca (0.287*); NEFA (0.195*) BHB (0.176*)	-
PI ²	Ca (0.299)**; Mg (0.195*); NEFA(0.189*) BHB (0.178*)	-

¹MFI- mean fluorescence intensity; ²Oxidative burst index = [(% oxidative burst positive cells) × (MFI)]/100; Alb- albumin levels in sera; Mg- magnesium levels in sera; Ca- calcium levels in sera; NEFA- non-esterified fatty acids levels in sera; BHB- β-hydroxybutyrate levels in sera; *significant differences (p < 0.05); **significant differences (p < 0.01).

Oxidative burst activity of neutrophils

The percentage of neutrophil granulocytes that exerted oxidative burst activity (OPC %) was not statistically significantly different between periods of sampling (Figure 5A). However, when comparing MFI-B values among sampling periods as well as OBI values (Figure 5B), significant differences were apparent. We noted a significant increase of MFI-B during the first week after calving. According to all this data there was no evidence of depressed ROS production.

Correlation of neutrophils oxidative burst activity with blood and metabolic profile parameters

Oxidative burst activity of neutrophils was also related to several metabolic parameters during transition period and early lactation. The levels of Ca²⁺, glucose, BHB, NEFA, TB and AST activity showed a positive correlation with the most of phagocytosis traits, particularly MFI and OBI. Negative correlation was noted between levels of urea and both, MFI and OBI. Glucose concentration was negatively correlated with OBI. Only variables with significant differences are presented (Table 2).

Discussion

High yielding dairy cows in the peripartum period might experience negative energy balance (NEB)

accompanied by immunosuppression. The latter is characterized by an impairment of neutrophil migration, phagocytosis and microbe killing capacity [21]. Immunosuppression is the mainstream conclusion of numerous authors, indicating that phagocytosis by PMNL gradually declines at the moment of calving and for two weeks afterwards [20,22]. The percentage of cells positive for phagocytosis was higher towards the end of our study period, but only numerical and biological differences in phagocytic capacity of neutrophils were observed. The values of MFI and PI increased until the second week after parturition. Various authors [23–26] also documented constant and high phagocytic capacity of neutrophils during the entire peripartum period.

In our study, the intensity of the ROS production was in accordance with the physiological needs of the transition period, as the most challenging period. The highest proportion of oxidative burst positive cells was observed during the week before parturition. Others also reported high oxidative burst activity of neutrophils in the week before and after calving followed by sharp decline from the 14th day onward [20]. The postpartum values for the percentage of oxidative burst positive cells were numerically lower, comparing them with results obtained in prepartum sampling periods, statistical significance was not found. The other two indicators of oxidative burst activity of

Table 2. Correlation of neutrophil oxidative burst activity and metabolic profile parameters before and after calving (total of 147 analyses) based on Pearson correlation coefficients.

Neutrophil activity	Peripartum period and early lactation (-3 to +6 weeks)	
	Positive (+) correlation	Negative (-) correlation
Phagoburst		
% of oxidative burst positive cells	Ca (0.202*); Glucose (0.167*)	
MFI ¹	Ca (0.164*); Mg (0.188*); NEFA (0.454**); BHB (0.534**); AST (0.381**); TB (0.23**)	Urea (0,223**); Glucose (0.171*)
OBI ²	Ca (0.212**); NEFA (0.423**); BHB (0.472**); AST (0.336**); TB (0.193*)	Urea (0.193*)

¹MFI- mean fluorescence intensity; ²Oxidative burst index = [(% oxidative burst positive cells) × (MFI)]/100; Ca- calcium levels in sera; Mg- magnesium levels in sera; NEFA- non-esterified fatty acids levels in sera; BHB- β-hydroxybutyrate levels in sera; AST- aspartate transaminase activity in sera; TB- total bilirubin levels in sera; *significant differences (p < 0.05); **significant differences (p < 0.01).

neutrophils (MFI-B and OBI) had higher values in the postpartum sampling period as compared to the values in prepartum sampling periods, which indicates high functional activity of neutrophils at postpartum period. The total average value for the percentage of cells with respiratory burst activation ($61.2 \pm 2.2\%$) was almost identical to the results of Meglia *et al.* [27]. It was evident that the intensity of ROS production is in accordance with the physiological needs and the most challenging period was characterized by a high functional activity of neutrophils.

NEB could lead to immunosuppression. The levels of NEFA and BHB are important risk factor for the impairment of chemotaxis phagocytosis as well as for the respiratory burst activity and efficient destruction of intruders in the body. Galvão and Santos [12] linked reduction of glycogen reserves in neutrophils with the development of uterine infections. Although the study was carried out during the transition period of dairy cows, notable NEB and significant changes in the functional activity of neutrophils were not observed. The values of biochemical and hematology parameters were mostly within the physiological ranges during the course of study. Enhanced mobilization of fat and accumulation of triglycerides in the liver is associated with decreased metabolic function of the liver [28]; however in this study, no signs of liver dysfunction were evident. The BHB levels, TB levels and AST activity also confirmed liver functionality. The levels of total proteins and albumin were stable and within physiological ranges through the whole study period. Maintaining a stable concentration of albumin is a reflection of preserved functional capacity of the liver [29]. The absence of the decreased neutrophil functional activity in our study could be explained by the fact that only NEFA levels were higher around parturition and all other biochemical parameters remained within physiological ranges. Concentrations of NEFA higher than 0.4mmol/L in the last 7 to 10 days of gestation are considered to be risk factor for many health disorders that may lead to untimely culling [30]. According to the [31] elevated NEFA led to the reduction of neutrophil activity. In our trial, the highest average concentration of NEFA was detected in the first week after calving (1.05 ± 0.50 mmol/L). These results might be linked to enhanced gluconeogenesis that occurs at the end of gestation [32,33], leading to an increased concentration of free fatty acids in the blood before calving [30] and reaching the highest values after calving [34,35]. These levels of NEFA obviously did not reach the levels that could influence the phagocytic ability of neutrophils, which corresponds with the

findings of previous study [36]. They reported that NEFA concentrations up to the 0.05mmol/L inhibit the respiratory burst of neutrophils but high concentrations of NEFA as a reflection of intense lipomobilization (2mmol/L), increased the intensity of the respiratory burst. The levels of BHB, total bilirubin and AST activity were within physiological ranges too. Only NEFA concentrations were moderately higher. As most of the changes were related for the period of one week before and one week after calving, the changes in the NEFA concentration also occurred in this period. NEFA values obtained in this study were higher from the week before until two weeks after calving. NEFA levels recorded in our study were in positive correlation with phagocytic activity (MFI and PI) as well as with the oxidative burst activity (OBI). BHB values between 1.2 and 1.4mmol/L indicate subclinical ketosis and an increased risk for health and reproductive disorders in cows in the first two weeks after calving [30]. Maximum levels of BHB in our trial were 0.82 ± 0.38 and 0.63 ± 0.40 in the first and second week after calving, respectively. These BHB concentrations observed in our study were below the concentration that were reported by Hoeben *et al.* [37] which were found to inhibit oxidative burst activity. Elbim and Lizard [38] did not prove the effect of BHB on the respiratory burst of neutrophils at concentrations from 1 to 10mmol/L. In our study BHB levels showed positive correlation with phagocytic activity (MFI and PI) as well as with oxidative burst activity (MFI and OBI). As a source of energy, BHB can be used by various tissues for the purpose of “saving” glucose [39], so we assume this can be the reason for positive correlation of BHB level with indicators of neutrophil activity. Glucose levels were within physiological range and did not show significant oscillations through the study period, although a negative correlation was established for MFI one week before and a week after calving

Results similar to ours were reported by Graungnard *et al.* [40]. They reported the increase of phagocytosis capacity in the overfed group from -14 to 7 days, which was explained by higher overall glucose concentration. In our study, the ration was balanced at 20% above NRC recommendation. In a recent study [41], the authors provided an insight into the potential immunomodulatory effects of feed additives (omega 3 - acids). An effect on the immune system was observed before parturition, which, according to the authors, might have been beneficial to the cow, to prepare for the metabolic changes post parturition. High oxidative burst values obtained in that study were similar with values obtained in our work. In that study, depression

of phagocytosis and respiratory burst activity was observed 7 days after calving, followed by rapid recovery starting from the day 14.

Likewise, the majority of the results of the hematological parameters showed decreased values at the second week after calving. Differences in the values that occurred before and after calving are in accordance with the recent results of other studies [42,43] and indicate that hematological parameters are a reflection of a normal physiological response to inflammation and stress around parturition.

During the trial, there were short-term oscillations (out of physiological ranges) in concentrations of Ca, P and Mg in the blood serum. However, at the same time, there were no evident signs of health disorders that might be correlated with the changes aforementioned minerals. Differences in the minerals concentrations identified at certain sampling periods may indicate active homeostatic mechanisms. Also, these results indicate that homeostatic mechanisms maintained energy balance without major consequences for the health status of dairy cows.

Conclusion

Disturbance of neutrophil function after calving was not a dominant phenomenon in our study. If all periods were summarized, the values of biochemical and hematological parameters were mostly within the physiological ranges during the course of study. The most prominent changes in the values of biochemical parameters occurred during the first two weeks after calving. None of the examined biochemical and hematological parameters had negative influence on phagocytosis. According to our results, there was no evidence of depressed ROS production. Notable NEB was not established and significant changes in the functional activity of neutrophils were not observed. These results indicate that homeostatic mechanisms maintained energy balance without major consequences for the health status of dairy cows.

Acknowledgements

This work was supported by a grant from scientific project TR 031071 (“Research on pharmacological characteristics of antimicrobial agents, introduction of new technological alternative methods of prophylactic with the purpose to improve control of infectious animal disease of farm animals”) of Ministry of Education and Science of Republic of Serbia.

Statement of ethical compliance

The experiment was performed in compliance with the Serbian Law on Animal Welfare (Official Gazette of the

Republic of Serbia, No. 41/09) and Ordinance on the conditions for registration for experimental animals and the keeping of such register, training programs on welfare for experimental animals, request forms for approval of conducting experiments on animals, standing, treatment and killing experimental animals and reproduction, circulation, or implementation experiments on animals (Official Gazette of the Republic of Serbia, No. 39/10).

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Corresponding author

Dr. Aleksandar Masic
Associate Professor,
Faculty of Ecological Agriculture, EDUCONS University, Serbia;
Vojvode Putnika 87, Sremska Kamenica 21208; Serbia
Phone: 021/ 4893-610,
Fax: 021/ 4893 -618)
e-mail: Aleksandar.Masic@educons.edu.rs

Conflict of interests: No conflict of interests is declared.

Annex – Supplementary Items

Supplementary Table 1. Values of biochemical parameters (mean ± SD/ median ± IQR) during peripartum period and early lactation in dairy cows (n = 21).

Parameter	SI units	Sampling time (weeks pre/postpartum)							Reference range (Merck)
		-3	-2	-1	+1	+2	+3	+6	
Total protein	g/L	70.10 ± 8.70	71.48 ± 7.62	71.91 ± 5.98	70.04 ± 6.79	69.87 ± 6.89	73.41 ± 6.04	75.37 ± 7.72	67-75
Albumin	g/L	28.90 ± 3.32	28.09 ± 2.48	28.08 ± 2.32	27.61 ± 3.34	27.54 ± 5.01	28.73 ± 3.87	30.55 ± 2.35	25-38
Globulin	g/L	41.20 ± 9.17	43.39 ± 7.42	43.84 ± 6.53	42.43 ± 5.62	42.32 ± 6.52	44.68 ± 5.90	44.82 ± 6.85	30-35
Urea*	mmol/L	3.96 ± 1.90	3.69 ± 1.49	4.30 ± 1.35	3.39 ± 1.73	3.32 ± 2.14	3.92 ± 1.85	4.56 ± 2.78	3.6-8.9
NEFA*	mmol/L	0.29 ± 0.15 ^{xy}	0.33 ± 0.34 ^{za}	0.56 ± 0.59 ^w	1.05 ± 0.50 ^{xzr}	0.81 ± 0.67 st	0.45 ± 0.57 ^u	0.14 ± 0.17 ^{wrtu}	< 0.7 mmol/L < 0.4 mmol/L**
BHB*	mmol/L	0.33 ± 0.29 ^x	0.30 ± 0.24 ^{yz}	0.30 ± 0.30 ^q	0.74 ± 0.43 ^{xy}	0.54 ± 0.46 ^{za}	0.41 ± 0.37	0.40 ± 0.14	< 1.0 mmol/L
Bilirubin*	µmol/L	1.87 ± 1.15 ^{ab}	2.29 ± 1.35	2.92 ± 2.09	3.96 ± 2.19 ^a	3.12 ± 1.67	2.91 ± 2.13 ^b	4.17 ± 2.20	0-27.4
Glucose	mmol/L	3.01 ± 0.46	3.08 ± 0.42	3.05 ± 0.41	2.72 ± 0.52	2.78 ± 0.53	2.98 ± 0.57	2.88 ± 0.62	2.2-5.6
AST*	U/L	78.60 ± 17.72 ^{axy}	72.20 ± 12.2 ^{zqw}	77.50 ± 28.15 ^t	97.50 ± 44.14 ^z	103.20 ± 31.42 ^{xq}	101.90 ± 30.3 ^{ywt}	86.73 ± 38.90	60-125
Ca	mmol/L	2.51 ± 0.32 ^{za}	2.79 ± 0.48	2.70 ± 0.37 ^b	2.75 ± 0.29 ^c	3.24 ± 0.96 ^{abc}	3.01 ± 0.49 ^a	2.96 ± 0.53	2.0-2.8
P*	mmol/L	2.03 ± 0.49	1.80 ± 0.39 ^x	1.84 ± 0.56	1.66 ± 0.59 ^y	1.74 ± 0.77	2.00 ± 0.96	2.50 ± 1.09 ^{xy}	1.8-2.6
Mg*	mmol/L	0.84 ± 0.43 ^{axy}	0.99 ± 0.34 ^{bc}	1.13 ± 0.44	1.11 ± 0.35	1.12 ± 0.88 ^a	1.46 ± 0.67 ^{xb}	1.44 ± 0.44 ^{yc}	0.6-1.2

* Median±IQR (interquartile range); ** during the last month of gestation; The same small letters a, b, c show a significant difference, p < 0.05; The same small letters x, y, z, q, w, r, t, u, show a significant difference, p < 0.01.

Supplementary Table 2. Values of hematological parameters (mean ± SD) during peripartum period and early lactation in dairy cows (n=21).

Parameter	SI units	Sampling time (weeks pre/postpartum)							Reference range (Merck)
		-3	-2	-1	+1	+2	+3	+6	
WBC	× 10 ⁹ /L	8.34 ± 1.56	8.14 ± 1.77	8.84 ± 2.61	8.25 ± 2.43	7.89 ± 2.48	7.56 ± 2.20	8.45 ± 2.21	4.0-12.0
Neutrophils	× 10 ⁹ /L	3.0 ± 0.98	4.0 ± 1.1	3.2 ± 2.11	2.9 ± 1.74	2.9 ± 1.83	2.7 ± 1.25	3.5 ± 1.46	0.6-4.0
Lymphocytes	× 10 ⁹ /L	5.06 ± 1.1	4.83 ± 1.04	4.51 ± 1.1	4.67 ± 1.16	4.64 ± 1.3	4.49 ± 1.34	4.57 ± 1.03	2.5-7.5
Lymphocytes	%	61.22 ± 8.77	58.38 ± 11.08	53.61 ± 13.58	57.83 ± 12.83	60.90 ± 14.79	61.28 ± 10.92	55.20 ± 8.48	45-75
Monocytes*	%	1.60 ± 3.10	1.20 ± 6.95	1.00 ± 7.55	1.00 ± 5.4	1.00 ± 8.2	1.00 ± 8.4	1.50 ± 8.45	0-8
Neutrophils*	%	37.20 ± 10.25	38.70 ± 10.45	41.30 ± 14.20	35.00 ± 19.60	34.60 ± 25.05	34.60 ± 8.35	36.90 ± 12.90	15-33
RBC	× 10 ¹² /L	6.67 ± 0.71	6.54 ± 0.71	6.54 ± 0.85	6.50 ± 0.95	5.97 ± 0.80	6.11 ± 0.84	6.04 ± 0.82	5.0-10.0
Hb	× 10 g/L	11.08 ± 1.12 ^{abc}	10.96 ± 1.07	10.93 ± 1.05	10.60 ± 1.11	10.01 ± 0.84 ^a	10.04 ± 1.05 ^b	10.07 ± 1.02 ^c	8-15
Hct/PCV	%	33.03 ± 4.07 ^x	32.56 ± 3.43 ^y	32.37 ± 3.63 ^z	32.76 ± 4.19 ^w	29.81 ± 2.67	29.87 ± 3.73	27.27 ± 3.10 ^{xyzw}	24-46
MCV	fL	50.05 ± 5.96 ^a	49.95 ± 5.25 ^b	49.90 ± 4.22 ^c	51.24 ± 4.35 ^x	49.33 ± 4.74	49.10 ± 3.85	45.48 ± 3.91 ^{abcx}	40-60
MCH	pg	16.77 ± 1.424	16.86 ± 1.311	16.87 ± 1.392	16.48 ± 1.639	16.57 ± 1.247	16.54 ± 1.297	16.79 ± 1.248	11-17
MCHC	× 10 g/L	33.68 ± 2.13 ^x	33.83 ± 1.77 ^y	33.83 ± 1.98 ^z	32.34 ± 2.25 ^w	33.68 ± 1.66 ^q	33.79 ± 2.56 ^r	37.00 ± 1.58 ^{xyzwrtq}	30-36

* Median ± IQR (interquartile range); ** during the last month of gestation; The same small letters a, b, c show a significant difference, p < 0.05; The same small letters x, y, z, q, w, r, t, u, show a significant difference, p < 0.01.