Investigation in blood Leukocytes and Neutrophils in Periparturient Dairy Cow

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ABSTRACT

Dairy cows are highly susceptible to infectious diseases, like mastitis, during the period around calving. Although factors contributing to increased susceptibility to infection have not been fully elucidated, impaired neutrophil recruitment to the site of infection and changes in the concentrations of some micronutrients related with the function of the immune defence has been implicated. Most of the current information is based on studies outside the Nordic countries where the conditions for dairy cows are different. Therefore, the aim of the study was to evaluate changes in blood concentrations of the vitamins A and E, the minerals Calcium (Ca), Phosphorous (P), And Magnesium (Mg), The Electrolytes Potassium (K) And Sodium (Na) And The Trace Elements Selenium (Se), Copper (Cu) And Zinc (Zn), As Well As Changes In Total And Differential White Blood Cell Counts (WBC) and expression of the adhesion molecules CD62L and CD18 on blood neutrophils in Swedish dairy cows during the period around calving. Blood samples were taken from 10 cows one month before expected calving, at calving and one month after calving. The results were mainly in line with reports from other countries. The concentrations of vitamins A and E, and of Zn, Ca and P decreased significantly at calving, while Se, Cu, and Na increased. Leukocytosis was detected at calving, mainly explained by neutrophilia, but also by monocytosis. The numbers of lymphocytes tended to decrease at the same time. The Mean Fluorescent Intensity (MFI) of CD62L and CD18 molecules on blood neutrophils remained constant over time. The proportion of CD62L+ neutrophils decreased significantly at calving. The animals were fed according to, or above, their requirements. Therefore, changes in blood levels of vitamins, minerals and trace elements were mainly in response to colostrum formation, changes in dry matter intake, and ruminal metabolism around calving. Decreased levels of vitamins A and E, and of Zn at calving might have negative implications for the functions of the immune defence. The lower proportion of CD62L+ neutrophils at calving may result in less migration of blood neutrophils into the tissues, and might contribute to the increased susceptibility to infections at this time.

Keywords: Dairy Cows; Periparturient Period; Leukocytes; Neutrophils; CD18; CD62L; Vitamin A; Vitamin E; Calcium; Phosphorous; Potassium; Sodium; Magnesium; Selenium; Copper; Zinc

INTRODUCTION

The susceptibility of dairy cows to infectious diseases, like mastitis, is higher during the period around calving than any other time. Host resistance mechanisms are usually depressed from approximately 3 weeks before calving until 3 weeks after calving [26]. Underlying mechanisms and factors have not been fully explained. However, many metabolic and hormonal changes take place during this period, which may contribute to the impaired immune defence [41,46,22]. Changes in white blood cell counts are observed around parturition, for example an increase in the numbers of circulating neutrophils (e.g. [11,21]). Neutrophils are considered the first line of cellular defence against pathogens. However, at calving, important neutrophil functions, like migration and phagocytosis, are impaired [16,21,37]. Reduced migration of blood neutrophils can be explained by a lower expression of the adhesion molecules CD62L (L-selectin) and CD11/CD18, which are of vital importance for their migration to the site of inflammation [29,24].

The nutritional status of the animals has been associated with the ability to resist infections. Reports have shown a depression in the blood levels of Calcium (Ca), Zinc (Zn), Magnesium (Mg), Phosphorous (P), Potassium (K), Selenium (Se), Vitamins A and E during the periparturient period [18,9,49,6,53]. Several of these nutrients are important for the immune system. Increased incidence of mastitis was reported at calving when the concentrations of vitamins A and E were decreased [3,27,32,43]. Selenium plays an important role in preventing impaired function of the immune response [43]. Neutrophils from Se-deficient animals were less capable of intracellular killing of mastitis pathogens [12,43]. Cu deficiencies have been shown to result in lowered bactericidal activities of blood leukocytes in cattle and sheep [19,52]. Moreover, [13] reported a higher proportion of uninfected quarters during the peripartum period in Holstein heifers after additional Cu supplementation. Zinc sufficiency has also been linked to proper immune functions, whereas deficiencies were related with irregular immunological profiles [17,34].

Most of the available information in this field is based on studies outside the Nordic countries where the conditions for dairy cows are different, for example in housing systems, feeding, climate and management. Therefore, the aim of this study was to evaluate leukocyte numbers and the expression of the adhesion molecules CD62L and CD18 on blood neutrophils, as well as blood vitamins A and E, the minerals Ca, P and Mg, the electrolytes K and Na, and the trace elements Se, Cu and Zn, during the periparturient period in Swedish dairy cows. This would also give baseline data for future studies in which different management routines could be compared.

MATERIALS AND METHODS

Animals

Ten healthy dairy cows of the Swedish Red and White breed at the university farm were monitored from one month before expected calving to one month after calving. The animals were in their second to sixth lactation and calved during March and April. They were fed with grass silage, concentrates and hay depending on their stage of lactation (Table (Table1).1). The animals were supplemented with 150 g/d of a commercial mineral and vitamin mix. Samples of hay, concentrate and silage were frozen at -20°C and analysed for contents of vitamins, minerals and trace elements. The total daily requirements and allotments of nutrients are given in Table Table22.

Table 1: Diet composition and estimated dry matter intake (DMI) of 10 dairy cows one month before expected calving, at calving, and one month after calving, expressed in kilograms of dry matter and in percentage (%) of the total diet.

	Before calving		At calving		After calving	
Concentrate	1	12.5	4	36.5	14	58.3
Grass hay	0[1]	0	0[1]	0	1	4.2
Grass silage	7	87.5	7	63.6	9	37.5
DMI	8	100	11	100	24	100

Table 2: Nutrient requirements according to NRC (National Research Council, 1989) and approximate daily allotments to ten dairy cows one month before expected calving (-1), at calving (0), and one month after calving (+1), calculated on a body weight of 600 kg, and an average milk production of 30 l/day one month after calving.

		Daily requirements (NRC)			Daily allotments		
		-1	0	+1	-1	0	+1
Energy							
ME1	Mcal	16.3	30.8	65.0	21.5	31.4	71.9
Protein	g	960	2090	3840	1237	1798	4068
Ca	g	31.2	84.7	139.2	83.9	94.7	151.1
K	g	52	110	216	217	243	409
Mg	g	12.8	27.5	48	21.2	26.6	49.0
Na	g	8	19.8	43.2	12.8	13.4	15.9
P	g	19.2	52.8	88.8	45.4	61.9	124.2
Cu	mg	80	110	240	246	291	473
Zn	mg	320	440	960	1140	1236	1650
Se	mg	2.4	3.3	7.2	7.1	7.2	7.9
Vit. A2	mg	32000	44000	76800	-2	-	-
Vit. E	mg	120	165	360	865	917	1127

1 Metabolizable energy.
2 The carotenoid content in the feedstuffs was not determined in this study. However, the estimated allotment was above NRC requirements.

Experimental Design

From each cow, jugular blood samples were collected in the morning, using Vacutainer® tubes (Becton Dickinson Vacutainer Systems, Meylan, France), one month before estimated calving, at calving (within 24 hours after calving) and one month after calving. Before sampling, the skin was cleaned with Milli-Q-water (Milli-Q, Millipore Corp., Bedford, MA, USA). Blood collected in a Zn-free vacutainer tube without additives was used for serum analyses of Zn, Cu, Ca, P, K, Na and Mg. Heparinized blood was used for separation of plasma and erythrocytes which was analysed for Se. Blood without additives was taken for serum vitamin E and vitamin A analysis. The tubes were centrifuged at 1500 g for 35 min to get plasma or serum, which was frozen at -20°C until analysis of the nutrients. Blood samples with EDTA added were taken for neutrophil immunostaining of CD18 and CD62L adhesion molecules, and for total and differential white blood cell counts.

Leukocyte Counts

Total and differential leukocyte counts were determined within 2 h using a Cell-DynR3500 (Abbott diagnostics, Abbott Laboratories, Abbott Park, IL, USA) according to standard procedures at the Department of Clinical Chemistry, Swedish University of Agricultural Sciences, Uppsala, Sweden.

Polymorphonuclear leukocyte immunostaining and flow cytometry analysis

For immunostaining with monoclonal antibodies (mAb), erythrocytes were lysed with ammonium chloride (NH_4Cl) before the staining procedure, and washed three times with phosphate buffered saline (PBS) without Ca and Mg. A double staining procedure was used to identify CD45+ leukocytes bearing the other markers of interest as described by [4]. The cell suspensions were labeled for flow cytometry with CD45 (clone CACTB51A, Veterinary Medical Research and Development (VMRD), Pullman, WA, USA), and either CD18 (clone BAQ30A, VMRD) or CD62L (clone BAQ92A, VMRD). Two secondary antibodies, goat anti-mouse IgG1 FITC (Caltag Laboratories, Burlingame, CA, USA), and goat anti-mouse IgG2a PE (Caltag), were used. The following controls were performed, blood without antibodies and blood with primary monoclonal antibodies CD45 (clone CACTB51A, VMRD) and a negative IgG1 isotype control (clone DAK-G01, DAKO, Glostrup, Denmark). Finally, the cell pellet was fixed in 200 µl of 1% paraformaldehyde in PBS and was stored in darkness at 4°C and analysed within a week. Before analysis, cells were washed twice and resuspended in PBS.

Stained cells were analysed on a FACStar Plus flow cytometer (Becton Dickinson Immunocytometry systems, Mountain View, CA, USA) with standard optical equipment using an argon ion laser at 200 mW tuned to 488 nm. The data were acquired with a FACstation, with the software Cellquest, version 1.2.2 (Becton Dickinson Immunocytometry Systems). Thirty thousand events were collected. The following parameters were obtained: forward light scatter (FSC), orthogonal light scatter (SSC), FITC fluorescence (FL1), and PE fluorescence (FL2). Leukocytes were identified by their expression of CD45, while their size (FSC) and granularity (SSC) identified polymorphonuclear leukocytes (PMNL). PMNL were gated to identify the proportions of CD18+ and CD62L+ cells. The discrimination between positive and negative cells was set using the isotype control. The mean fluorescent intensity (MFI) of each cell in FL1 was determined using quantum beads (Flow Cytometry Standards Corporation, San Juan, Puerto Rico).

Analysis of Vitamins, Minerals and Trace Elements

Vitamin A and E were extracted from the serum samples with hexan. The separation was done by High Performance Liquid Chromatography (HPLC) on a C18 colonn. Vitamin A and E were determined by using ultraviolet and fluorescence detection, respectively according to standard procedures at the Department of Chemistry, National Veterinary Institute, Uppsala, Sweden.

Serum samples were diluted (1:10) with ultrapure water (Milli-Q). The determination of Ca, Cu, K, Mg, Na and Zn was performed using inductively coupled plasma emission spectrometry (ICP-AES, Jobin Yvon 238 emission-spectrometer, Instruments S.A., Division Jobin Yvon, Longjuemeau, France) with set-up and conditions according to the method accredited by SWEDAC (Swedish Board for Accreditation and Conformity Assessment). Serum inorganic phosphate (P) was determined according to standard procedures at the Department of Clinical Chemistry, Swedish University of Agricultural Sciences, Uppsala, Sweden.

The Se concentrations in the plasma and erythrocyte fractions were determined with flow injection hydrid generation atomic absorption spectrometry (FI-HG-AAS) after wet digestion of the biological material with a mixture of oxidizing acids [8]. Selenium content in whole blood was calculated from plasma Se and erythrocyte Se assuming an average hematocrit content of 35% [38].

STATISTICAL ANALYSIS

Analyses of variance for the concentrations of nutrients and leukocytes, and the proportions and MFI for the neutrophil adhesion molecules were done using the General Linear Model (SAS Institute Inc., Cary, NC, USA). The effects of cow and period were included in the model. Mean fluorescent intensity for CD18 and CD62L were log-transformed. The results are presented as least square means ± standard error of the mean (LSM ± SEM). Probabilities less than 0.05 were considered significant.

RESULTS

Total and Differential Blood Leukocytes

The total white blood cell counts (WBC) were significantly ($p < 0.05$) higher at parturition than before and after calving (Figure (Figure1).1). This was mainly due to a significant increase in the numbers of neutrophils reaching values over the normal range (0.6–4.0 × 109/l) in 6 cows, and to a lesser extent, to a significant increase in the numbers of monocytes at this time point (Figure (Figure1).1). The numbers of lymphocytes did not differ significantly between sampling occasions, but was lower than the normal range (2.5–7.5 × 109/l) in 8 cows at calving (Figure (Figure11).

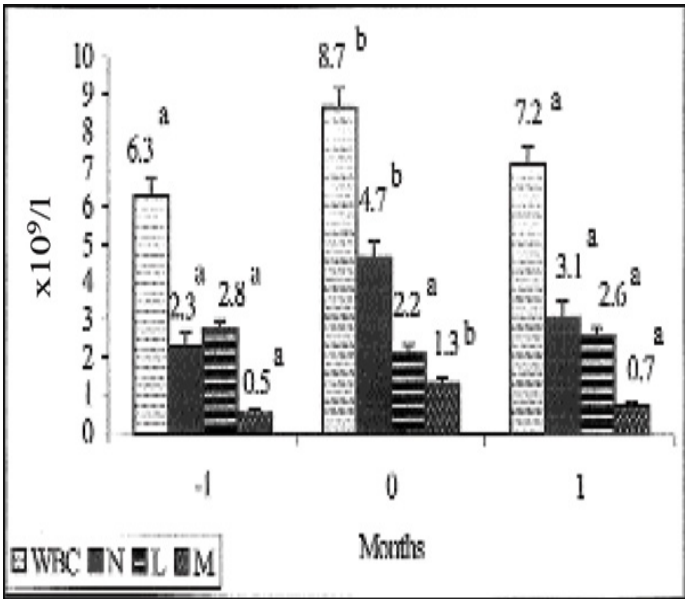

Figure 1: Numbers (× 109/l, LSM ± SEM) of white blood cells (WBC), neutrophils (N), lymphocytes (L), and monocytes (M) in blood samples taken one month before expected calving (-1), at calving (0), and one month after calving (+1) from ten dairy cows. Values with different letters within each parameter differ significantly ($p < 0.05$).

NEUTROPHIL ADHESION MOLECULES

Most neutrophils were positive for both CD18 and CD62L (Figure (Figure2).2). The proportion of CD18+ neutrophils remained fairly constant, but was significantly ($p < 0.05$) higher after calving than before calving. In contrast, the proportion of CD62L+ neutrophils decreased significantly ($p < 0.05$) at calving. A fairly large variation in proportion positive cells at calving explained the large standard error of means before and after calving. The log MFI for CD62L and CD18 on blood neutrophils was, on average, 11.63 ± 0.09 and 11.90 ± 0.11 at calving, respectively, and did not change significantly during the sampling period (data not shown).

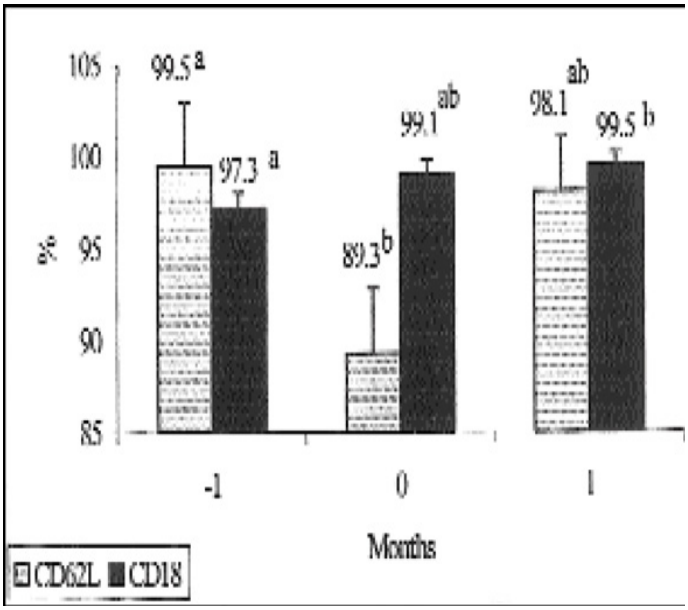

Figure 2: Proportions (%, LSM ± SEM) of CD62L+ and CD18+- blood neutrophils in blood samples taken one month before expected calving (-1), at calving (0), and one month after calving (+1) from ten dairy cows. Values with different letters within each parameter differ significantly ($p < 0.05$).

VITAMINS A AND E

The serum concentrations of vitamins A and E are shown in Figure Figure3.3. The level of vitamin A changed significantly ($p < 0.001$) over time. It was significantly lower at parturition than before or after calving, reaching values (0.23 ± 0.02 mg/l) considered marginal [33]. The levels of vitamin E did also tend ($p = 0.065$) to decrease at calving and was significantly ($p < 0.05$) higher one month after calving than before and at calving.

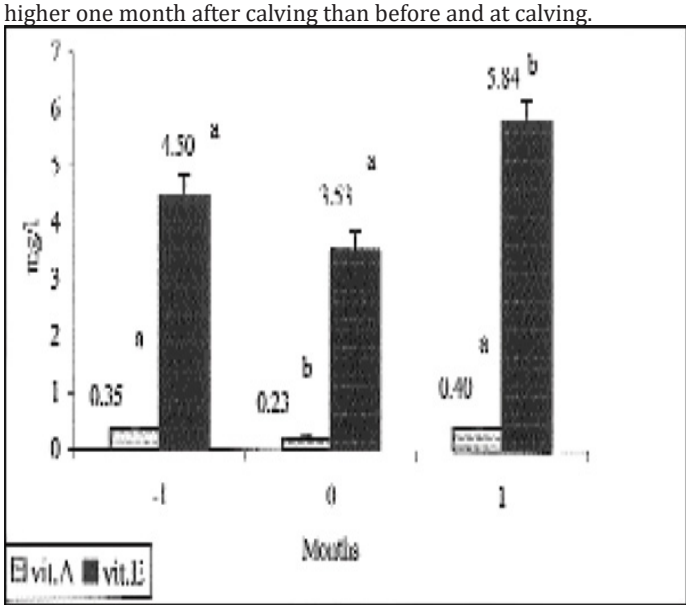

Figure 3: Serum concentrations of vitamins A and E (mg/l, LSM ± SEM) in blood samples taken one month before expected calving (-1), at calving (0), and one month after calving (+1) from ten dairy cows. Samples with different letters within each parameter differ significantly ($p < 0.05$).

Minerals, Electrolytes and Trace Elements

The serum concentrations of Ca, P, K, Cu, Zn and Se are shown in Figures Figures4,4, ,5,5, ,6.6. The levels of Ca and Zn were significantly ($p < 0.05$) lowered at calving, reaching levels just under the reference values 2.1–2.7 mmol/l and 11–23 µmol/l, respectively. In contrast, the serum concentration of Cu was significantly ($p < 0.05$) higher at calving and one month after calving ($p < 0.001$) compared with before calving. The P levels decreased significantly ($p < 0.05$) at calving and remained depressed after calving compared with before calving. The K levels did not decrease significantly ($p < 0.05$) until after calving, reaching values under the normal reference range of 4.0–5.6 mmol/l. The concentration of plasma, erythrocytic and whole blood Se changed slightly, but significantly, over time (Figure (Figure6).6). Plasma Se was significantly ($p < 0.05$) higher at calving compared with after calving, whereas erythrocytic Se was significantly ($p < 0.05$) higher at calving than before calving. Whole blood Se was significantly ($p < 0.001$) higher at parturition than before and after calving.

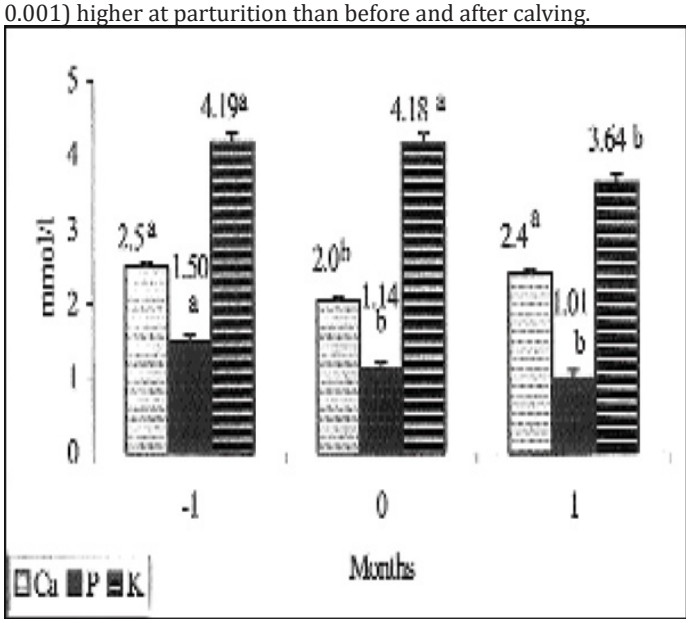

Figure 4: Serum concentrations of calcium (Ca), phosphorous (P), and potassium (K) (mmol/l, LSM ± SEM) in blood samples taken one month before expected calving (-1), at calving (0), and one month after calving (+1) from ten dairy cows. Values with different letters within each parameter differ significantly ($p < 0.05$).

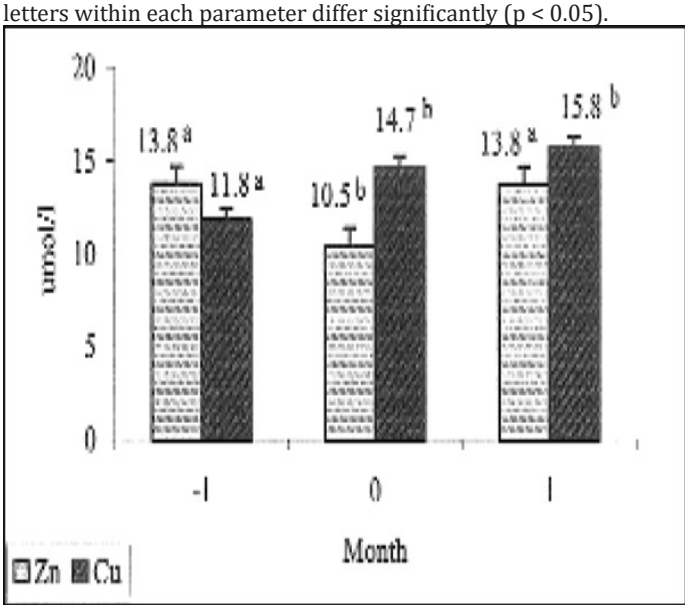

Figure 5: Serum concentrations of zinc (Zn), and copper (Cu) (µmol/l, LSM ± SEM) in blood samples taken one month before expected calving (-1), at calving (0), and one month after calving (+1) from ten dairy cows. Values with different letters within each parameter differ significantly ($p < 0.05$).

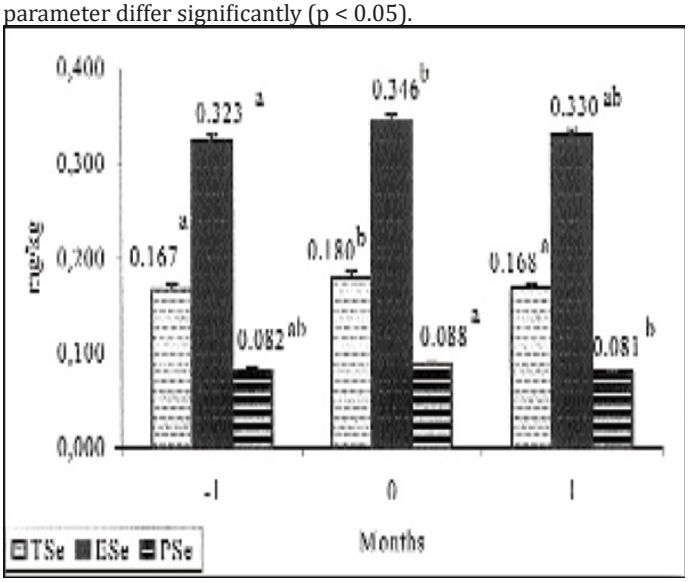

Figure 6: Whole blood Se (TSe), erythrocyte (ESe), and plasma selenium (PSe) concentrations (mg/kg, LSM ± SEM) in blood samples taken one month before expected calving (-1), at calving (0), and one month after calving (+1) from ten dairy cows. Values with different letters within each parameter differ significantly ($p < 0.05$).

The Na concentrations were 138.3 ± 1.2, 142 ± 1.2 and 138 ± 1.2 mmol/l before, at and after calving, respectively. The value at calving was significantly ($p < 0.05$) higher than at the other time points. The Mg concentrations remained fairly constant over time, at approximately 1.05 ± 0.05 mmol/l.

DISCUSSION

In agreement with earlier studies (e.g. [11,21]), we detected a significant increase in the numbers of WBC at calving. This was mainly due to an increase in the numbers of circulating neutrophils, and to a less extent, an increase in monocytes. At calving, the levels of corticosteroids are elevated [41,11]. Corticosteroids induce neutrophilia by an increased output of neutrophils from the bone marrow, by neutrophil demargination from the blood vessel wall, or by a combination of the two [36,24]. According Our results to [24], the neutrophil expression of CD18 increases, while the expression of CD62L decreases at calving. Such changes were not observed in this study as the expression of CD62L and CD18 remained constant over time. However, we observed a depression in the proportion of CD62L+ neutrophils at calving, in accordance with [24]. Fewer cells expressing this molecule means that the marginating pool of neutrophils, rolling along the vessel wall, will shift to the main blood flow stream contributing to the leukocytosis. As a result, fewer neutrophils are able to migrate into the tissues. In agreement with [39], we found that the numbers of blood lymphocytes were reduced at calving. [1] presented the hypothesis that lymphocytes migrate in a different manner than neutrophils, suggesting that the high levels of cortisol detected at calving do not affect the adhesion molecules of lymphocytes and therefore they can migrate into the tissues. have shown a marked decline at calving in serum concentrations of vitamins A and E, and in Zn in agreement with earlier reports [18,9,49,53]. A drop in the serum concentrations of these nutrients is associated with impaired immune functions and a higher incidence of diseases, like mastitis [18,34,27,43].

The drop in serum concentrations of vitamins A and E is largely due to colostrum formation [9], but can also be due to changes in dry matter intake and ruminal metabolism [50]. Moreover, storage and season can have negative effects on the amount of vitamins A and E in the feedstuffs [9,28,33]. Dry matter intake (DMI) can drop remarkably during the week before calving [2,10]. As a result, reduced blood concentrations of nutrients can be expected, especially as the nutrient demands to initiate milk synthesis is increasing. However, [31] reported less reduction in DMI before calving in Swedish dairy cows fed high quality feedstuffs. Ruminal metabolism has been implicated in the destruction of vitamin E [40], but others have suggested that ruminal vitamin E metabolism is essentially nil [25,51]. Vitamin E in blood is present mainly as a component of lipoproteins. As parturition approaches, the liver secretion of lipoproteins decreases. As a consequence, its transport capacity of vitamin E is lowered [14]. However, the ruminal destruction of vitamin A can be substantial and increases as the level of concentrates in the diet is elevated [35,51].

The significant drop in serum Zn concentration reported at calving, is most likely a consequence of colostrum formation [9] and increased stress e.g. in association with an acute phase response due to inflammatory reactions in the uterus. Stress induces synthesis of metallothionein, a protein associated with Zn distribution. As a consequence, Zn is redistributed from blood to other tissues, such as the liver [44,53]. Physiological fluctuations occur immediately before and after calving in the blood levels of Ca, P, K and Na [6]. Blood levels of Ca and P is expected to decrease at calving due to the large demand of colostrum and milk production. In agreement with [7], we detected a reduced blood P concentration one month after calving. There is an inverse relationship between milk production and plasma P concentration [7]. The K values were also depressed one month after calving, which might be related with K being the major cation secreted into the milk of cattle [45].

The blood Cu status undergoes several changes during the periparturient period. The lower value before calving could be due to the drainage by the fetal liver [53]. In contrast to other reports [15,53], an increased blood level of Cu was detected at calving in this study. [47] suggest that cattle undergoing stressful periods have increased blood levels of Cu and ceruloplasmin, as Cu transport protein. Ceruloplasmin is considered an acute phase protein and its concentration increase in response to injury, infections and inflammation [5]. This might be one reason for the increased blood level of this nutrient, as calving is considered a stressful period with tissue damages for example in the uterus.

There is a relationship between the Se status of the animals around parturition and the functions of the immune system and disease resistance [12,43]. From these studies it can be concluded that beneficial effects of Se supplementation occur only when the animals are Se deficient. Whole blood Se levels in the range

of 0.1–0.2 mg/l could be considered optimal from immunological standpoint [23,20]. In this study, whole blood Se concentrations were in the range of 0.167–0.180 mg/kg, i.e. according to recommendations. [49] hypothesised that the increase in the level of Se at calving may be related to the high fragility of the red blood cells detected at calving.

In conclusion, the results obtained under Swedish conditions were mainly in line with earlier reports. At calving, leukocytosis due to neutrophilia and monocytosis was detected. A lower proportion of CD62L+neutrophils at calving suggests that fewer of these cells can migrate into the tissues with negative consequences for the defence against infections. Moreover, reduced concentrations of vitamins A and E, and the trace element Zn, were observed at this time. This can also have negative effects on the functions of the immune system resulting in increased susceptibility to diseases, such as mastitis. Despite the fact that vitamin A was fed according to recommendations, and vitamin E above recommendations, the levels of both nutrients decreased at calving. Vitamin A levels dropped below the normal reference value, while vitamin E levels remained within the normal range. Several authors [42,27,48] reported improvements in milk production, immune functions and mammary gland health when additional vitamins A and E were given compared with NRC [30]recommendations. Results from the present study, in combination with aforementioned data, suggest that the NRC vitamin A and E recommendations may not be adequate, at least not around calving. However, further studies are needed to evaluate whether the low blood values reflect a true body deficiency and the importance of decreased absorption of vitamins during this period.

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