Changes in some Blood Micronutrients, Leukocytes and Neutrophil Expression of Adhesion Molecules in Periparturient Dairy Cows

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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 (Mallard et al. 1998). 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 (Smith et al. 1973, Van Kampen & Mallard 1997, Kehrli et al. 1998).

Changes in white blood cell counts are observed around parturition, for example an increase in the numbers of circulating neutrophils (e.g. Guidry et al. 1976, Kehrli et al. 1989). Neutrophils are considered the first line of cellular defence against pathogens. However, at calving, important neutrophil functions, like migration and phagocytosis, are impaired (Hill 1981, Kehrli et al. 1989, Saad et al. 1989). 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 (Nagahata et al. 1995, Lee and Kehrli 1998).

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 (Johnston and Chew 1984, Goff and Stabel 1990, Weiss et al. 1990, Xin et al. 1993). 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 (Chew et al. 1982, Michal et al. 1994, Politis et al. 1995, Smith et al. 1997). Selenium plays an important role in preventing impaired function of the immune response (Smith et al. 1997). Neutrophils from Se-deficient animals were less capable of intracellular killing of mastitis pathogens (Gyang et al. 1984, Smith et al. 1997). Cu deficiencies have been shown to result in lowered bactericidal activities of blood leukocytes in cattle and sheep (Jones and Suttle 1981, Xin et al. 1991). Moreover, Harmon et al. (1998) 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 (Hutcheson 1989, Reddy and Frey 1990).

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 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 2.
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-Dyn®3500 (Abbott diagnostics, Abbott Laboratories, Abbott Park, IL, USA) according to standard procedures at the Department of Clinical Chemis-

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 |
|------------|---------------|-----------|--------------|
| Kg         | %             | Kg        | %            | Kg           | %          |
| Concentrate| 1             | 3.5       | 4            | 36.4         | 14         | 58.3       |
| Grass hay  | 0             | 0         | 0            | 0            | 1          | 4.2        |
| Grass silage| 7             | 87.5      | 7            | 63.6         | 9          | 37.5       |
| DMI        | 8             | 100       | 11           | 100          | 24         | 100        |

1 The cows had access to straw

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 ME 1               | 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. A 2                 | 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.
Polymorphonuclear leukocyte immunostaining and flow cytometry analysis

For immunostaining with monoclonal antibodies (mAb), erythrocytes were lysed with ammonium chloride (NH₄Cl) 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 Colditz et al. (1996). 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 column. 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, Longjumeau, 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 ox-
Idizing acids (Galgan and Frank 1993). Selenium content in whole blood was calculated from plasma Se and erythrocyte Se assuming an average hematocrit content of 35% (Schalm 1986).

**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 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 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 1).

Neutrophil adhesion molecules
Most neutrophils were positive for both CD18 and CD62L (Figure 2). The proportion of CD18+ neutrophils remained fairly constant, but was significantly (p<0.05) higher after calving than before calving. In contrast, the propor-

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).

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).
tion 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).

Vitamins A and E

The serum concentrations of vitamins A and E are shown in Figure 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 (Puls 1995). 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.
Minerals, electrolytes and trace elements

The serum concentrations of Ca, P, K, Cu, Zn and Se are shown in Figures 4-6. 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 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. 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. Guidry et al. 1976, Kehrli et al. 1989), 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 (Smith et al. 1973, Guidry et al. 1976). 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 (Roth et al. 1982, Lee and Kehrli 1998). According to Lee & Kehrli (1998), 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 Lee & Kehrli (1998). 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 Shafer-Weaver et al. (1996), we found that the numbers of blood lymphocytes were reduced at calving. Alon et al. (1995) 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.

Our results have shown a marked decline at calving in serum concentrations of vitamins A and E, and in Zn in agreement with earlier reports (Johnston & Chew 1984, Goff & Stabel 1990, Weiss et al. 1990, Xin et al. 1993). A drop in the serum concentrations of these nutrients is associated with impaired immune functions and a higher incidence of diseases, like mastitis (Johnston & Chew 1984, Reddy & Frey 1990, Michal et al. 1994, Smith et al. 1997). The drop in serum concentrations of vitamins A and E is largely due to colostrum formation (Goff & Stabel 1990), but can also be due to changes in dry matter intake and ruminal metabolism (Weiss et al. 1994). Moreover, storage and season can have negative effects on the amount of vitamins A and E in the feedstuffs.
(Goff & Stabel 1990, Miller et al. 1995, Puls 1994). Dry matter intake (DMI) can drop remarkably during the week before calving (Bertics et al. 1992, Grummer et al. 1995). As a result, reduced blood concentrations of nutrients can be expected, especially as the nutrient demands to initiate milk synthesis is increasing. However, Olsson (1996) 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 (Shin & Owens 1990), but others have suggested that ruminal vitamin E metabolism is essentially nil (Leedle et al. 1993, Weiss et al. 1995). 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 (Herdt & Stowe 1991). However, the ruminal destruction of vitamin A can be substantial and increases as the level of concentrates in the diet is elevated (Rode et al. 1990, Weiss et al. 1995).

The significant drop in serum Zn concentration reported at calving, is most likely a consequence of colostrum formation (Goff & Stabel 1990) 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 (Spears et al. 1991, Xin et al. 1993). Physiological fluctuations occur immediately before and after calving in the blood levels of Ca, P, K and Na (Dukes 1993). 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 Forar et al. (1982), we detected a reduced blood P concentration one month after calving. There is an inverse relationship between milk production and plasma P concentration (Forar et al. 1982). 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 (Underwood & Suttle 1999).

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 (Xin et al. 1993). In contrast to other reports (Hidiroglou & Knipfel 1981, Xin et al. 1993), an increased blood level of Cu was detected at calving in this study. Ward & Spears (1999) 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 (Conner et al. 1986). 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 (Gyang et al. 1984, Smith et al. 1997). 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 (Koller et al. 1983, Jukola et al. 1996). In this study, whole blood Se concentrations were in the range of 0.167-0.180 mg/kg, i.e. according to recommendations. Weiss et al. (1990) 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 (Smith et al. 1984, Michal et al. 1994, Weiss 1998) reported improvements in milk production, immune functions and mammary gland health when additional vitamins A and E were given compared with NRC (National Research Council 1989) 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.

Acknowledgements
The authors thank Inga-Lena Örde-Öström, Gabor Sellei, and Anna Stepinska, National Veterinary Institute (SVA), Uppsala, Sweden for skilful technical assistance.

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Sammanfattning

Förändringar i vissa mikronäringsämnen, leukocyter och uttryck av adhesionsmolekyler på neutrofiler i blodet hos peripartala mjölkor.

Mjölkor är mycket känsliga för infektionssjukdomar, som mastit, under perioden runt kalvningen. Det är inte helt klargt vilka faktorer som bidrar till den ökade infektionskänsligheten men försämrad rekrytering av neutrofiler till infektionsplatsen och förändringar i koncentrationen av vissa mikroelement har troligen betydelse. De flesta befintliga uppgifter baseras på studier gjorda utanför Norden där förhållandena för mjölkor är annorlunda. Syftet med denna studie var därför att undersöka förändringar i blodkoncentrationen av vitaminerna A och E, mineralerna kalcium (Ca), fosfor (P) och magnesium (Mg), elektrolyterna kaliyum (K) och natrium (Na) samt spårämnen selen (Se), koppar (Cu) och zink (Zn) hos svenska mjölkor under perioden runt kalvning. Dessutom undersöktes förändringar i antalet vita blodkroppar och uttrycket av adhesionsmolekylerna CD62L och CD18 på blodneutrofiler. Resultaten stämde huvudsakligen med tidigare rapporter från andra länder. Koncentrationen av vitaminerna A och E, Zn, Ca och P minskade signifikant vid kalvning medan Se, Cu och Na ökade. Leukocyten sågs vid kalvning huvudsakligen orsakat av neutrofili men också till viss del monocyten. Antalet lymfocyter tenderade att minska vid samma tidpunkt. Medelvärdet av fluorescensintensiteten för CD62L- och CD18-molekylerna på blodneutrofiler var konstant över tiden. Andelen CD62L+ neutrofiler minskade dock signifikant vid kalvningen. Djuren utförde i enlighet med eller över deras behov varför förändringarna i blodnivåerna av vitaminer, mineraler och spårämnen huvudsakligt var ett resultat av kolostrumproduktion, samt förändringar i torsubstansintag och vämst process runt kalvningen. Minskade nivåer av vitaminerna A och E och av Zn vid kalvning kan ha negativ effekt på immunförsvarets funktion. Den lägre andelen CD62L+ neutrofiler vid kalvning kan resultera i en försämrad mi-

(Received August 14, 2000, accepted October 20, 2000).

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Acta vet. scand. vol. 42 no. 1, 2001