

Nutrition and Immune Response in Periparturient Dairy Cows

with Emphasis on Micronutrients

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Abstract

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The general aim of the thesis was to increase the knowledge about the complex changes in, and interactions between, nutrition and immune system in dairy cows during the period around calving with emphasis on micronutrients. The specific aims were to study changes in blood concentrations of selected micronutrients and leukocytes, evaluate the effects of feeding intensity during the dry period on blood leukocyte numbers and functions, evaluate if a mid dry period blood sample can predict the micronutrient concentration at calving, and to compare the α -tocopherol concentration and stereoisomer composition in blood and milk after supplementation of synthetic or natural vitamin E, in periparturient dairy cows.

Blood samples were taken around calving from 10 dairy cows fed according to Swedish recommendations to evaluate the blood concentrations of vitamins, minerals and trace elements. At calving, leukocytosis due to neutrophilia and monocytosis was detected, as well as a decreased proportion of neutrophils with adhesion molecules. Moreover, reduced concentrations of vitamins A and E, and zinc (Zn) were observed at calving.

Twenty-three dairy cows were fed three different levels (low (L), medium (M) and high (H)) of a total mixed ration during the dry period. Blood samples were taken from 8 weeks before to 8 weeks after calving. Dry period diet had small effects on leukocyte numbers, and had no effects on neutrophil functions and disease incidence. However, an increase in the proportion of B-cells and a decrease in WC1⁺ T-cells were observed after calving in H and L cows, but not in M cows. The weeks around calving were characterised by neutrophilia, eosinopenia, lymphopenia and monocytosis, and the proportions of certain lymphocyte sub-populations increased in early lactation.

The concentrations of vitamins A and E, selenium (Se) and Zn in blood was measured at several time points from one month before to one month after calving in 23 dairy cows fed three different feeding regimens during the dry period. The concentrations of vitamin A and E, and Zn decreased at calving, and Se was lower during the dry period than in early lactation. The concentrations of vitamin E and Se in the mid dry period sample predicted the occurrence of values considered marginal or deficient at calving.

The effect of supplementation of 36 dairy cows with natural or synthetic vitamin around calving on the α -tocopherol concentration and stereoisomer composition in blood and milk was compared. The α -tocopherol concentration in blood was higher in the group fed RRR- α -tocopheryl acetate than in the groups fed all-*rac*- α -tocopheryl acetate, RRR- α -tocopherol or no supplement. A significant effect of time was also observed with lowest values at calving. The α -tocopherol concentration in milk was not affected by treatments, but was higher in colostrum than in milk. The proportion of the RRR-isomer was lower in the group fed synthetic vitamin E than in the other groups both in plasma and milk.

Key words: dairy cows, micronutrients, immune response, periparturient period, vitamin E, Se, Zn, leukocytes, lymphocytes, neutrophils

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....hay hombres que de su ciencia tienen la cabeza llena; hay sabios de todas menas mas digo sin ser muy ducho: es mejor que aprender mucho el aprender cosas buenas....

Martin Fierro

To Silvina with all my love and
our children Tomás and Martina.

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Papers I-IV

The present thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

- I. Meglia G. E., Johannisson A., Petersson L., & Persson Waller K. 2001. Changes in some blood micronutrients, leukocytes and neutrophil expression of adhesion molecules in periparturient dairy cows. *Acta Veterinaria Scandinavica* 42, 139 – 150.
- II. Meglia, G. E., Johannisson, A., Agenäs, S., Holtenius, K., & Persson Waller K. 2004. Effects of feeding intensity during the dry period on leukocyte and lymphocyte sub-populations, neutrophil function and health in periparturient dairy cows. *The Veterinary Journal*, in press.
- III. Meglia, G. E., Holtenius, K., Petersson, L., Öhagen, P., & Persson Waller, K. 2004. Prediction of vitamin A, vitamin E, selenium, and zinc status of periparturient dairy cows using blood sampling during the mid dry period. *Acta Veterinaria Scandinavica*, in press.
- IV. Meglia, G. E., Jensen, S. K., Lauridsen, C., & Persson Waller, K. 2004. α -Tocopherol concentration and stereoisomer composition in plasma and milk from dairy cows fed natural or synthetic vitamin E around calving. Manuscript.

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Abbreviations

α -TTP	alpha tocopherol transfer protein
A	adequate concentration
ADCC	antibody dependent cell-mediated cytotoxicity
APC	antigen presenting cells
APP	acute phase protein
Ca	calcium
Co	cobalt
CR	complement receptor
Cu	copper
Cu-Zn SOD	Cu-Zn superoxide dismutase
DIM	days in milk
DM	dry matter
DMI	dry matter intake
FITC	fluorescein isothiocyanate
FSC	forward light scatter
H	high feeding intensity group
H ₂ O ₂	hydrogen peroxide
HPLC	high performance liquid chromatography
ICP-AES	inductively coupled plasma atomic emission spectrometry
IFN- γ	interferon gamma
Ig	immunoglobulin
IL	interleukin
IU	international unit
K	potassium
KOH	potassium hydroxide
L	low feeding intensity group
LPS	lipopolysaccharide
M	medium feeding intensity group
Mcal	megacalories
MD	marginal or deficient concentration
Mg	magnesium
MHC-II	major histocompatibility complex class II
Na	sodium
NEFA	non-esterified fatty acids
NE _L	net energy of lactation
NK	natural killer cells
NRC	national research council
O ₂ ⁻	superoxide
PAMPs	pathogen-associated molecular patterns
PE	phycoerythrin
PUFA	polyunsaturated fatty acids
S	sulphur
SAA	serum amyloid A
SSC	orthogonal light scatter
SCC	somatic cell count

Se	selenium
TFG- β	transforming growth factor beta
Th1	cell mediated immune response
Th2	humoral immune response
TLRs	toll-like receptors
TMR	total mixed ratio
WBC	white blood cells
Zn	zinc

Background

Introduction

Through genetic selection and improved feeding and management, the milk yield of the modern dairy cows increase 2-3% annually (Hutjens, 1996). As the management becomes more intensive and the number of cows per herd increases, the risk of metabolic disturbances may increase, which can have negative consequences on animal health (Goff & Horst, 1997; Rukkwamsuk et al., 1999). Metabolic disturbances are most common in early lactation and are often associated with changes in management, feeding routines and diet composition, but also in hormonal levels, occurring around parturition (Smith et al., 1973; Bell & Bauman, 1997; Kehrli et al. 1999; Persson Waller, 2000). This can lead to an increased incidence of metabolic diseases like ketosis, fatty liver and reproductive associated problems (Markusfeld, 1985; Goff & Horst, 1997; Rukkwamsuk et al., 1999). However, the incidence of infectious diseases can also increase due to suppression of immune functions during this period. Important infectious diseases occurring during the periparturient period are mastitis and endometritis (Eberhart, 1986; Gröhn & Rajala-Schultz, 2000). Mastitis is the most common disease in dairy cows in many countries with serious economic consequences mainly due to reduced milk production and changes in milk quality (Smith & Hogan, 2001).

The feeding during the dry period is very important for the performance and general health of the dairy cows during early lactation. Mismanagement of the late gestation diet can have a negative effect on the dry matter intake (DMI) during the beginning of lactation, predisposing the animal to both metabolic and infectious diseases (Østergaard & Sorensen, 1998; Rukkwamsuk et al., 1999). A balanced supply of micronutrients, such as vitamins A and E, and the trace elements selenium (Se) and zinc (Zn), is also of great importance as deficiency of these micronutrients have been associated with an increased incidence of diseases (Kellogg, 1990; Hemingway, 1999, Weiss, 2002). The importance of vitamin E for a well-functioning immune response around parturition has lately been given increasing interest (Weiss, 1998; NRC, 2001). At present, synthetic vitamin E (all *rac*- α -tocopheryl acetate) is mostly used for supplementation of the diet. However, the natural form of vitamin E (RRR- α -tocopheryl) has been shown to have higher biopotency than the synthetic one in cattle and pigs (Hidiroglou et al., 1988; Mahan et al., 2000). This finding might be especially important when supplementing dairy cows around calving.

The bovine immune system

General overview

The immune system in ruminants can be divided in the innate and the acquired immune system. Innate immunity is the predominant defence during early stages of infections. It is activated by antigens, but the response is not amplified by repeated exposure to the same antigen (Tizard, 2000). By contrast, the acquired, or adaptive, immune system recognizes specific antigen determinants and is mediated by antigen presenting cells (APC). If the host encounters the same antigen more

than once, an enhanced immune reactivity occur due to immunological memory (Janeway et al., 2001; Sordillo & Streicher, 2002). Both parts of the immune system have the capacity to recognize conserved components of pathogens called pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), peptidoglycan and bacterial DNA (Hornef et al., 2002). The host cell recognition of these molecules relies on a number of membrane receptors, i.e. the Toll-like receptors (TLRs), which provide cellular signalling during the initiation of the immune response (Medzhitov et al., 1997; Janeway et al., 2001).

Leukocytes and various soluble factors are important components in the immune response. Among those, neutrophils, macrophages, natural killer (NK) cells and soluble factors such as complement and lysozyme mediates the innate immune response, whereas lymphocytes, macrophages and soluble components such as immunoglobulins compose the specific immune response (Sordillo et al., 1997; Tizard, 2000).

The leukocytes, or white blood cells (WBC), are produced in the bone marrow. Most of them, with the exception of some types of lymphocytic and dendritic cells, also mature in the same place. When released from the bone marrow they patrol the body circulating in blood, lymph and different tissues.

Neutrophils

Neutrophils are considered the first cellular line against infections (Craven & Williams, 1985; Tizard, 2000), and constitute between 20-30% of the total blood leukocytes in ruminants. The major functions of neutrophils are phagocytosis and killing of invading pathogens (Tizard, 2000). To be effectively phagocytosed by neutrophils, the invading pathogens have to be opsonised by complement fractions (C3b) or immunoglobulins (IgG₂, IgM). Immunoglobulins recognize bacteria by the Fab region and bind to Fc receptors in the surface of the neutrophils, whereas complement components once has been attached to the bacteria bind to neutrophils via CR1 and CR3 receptors (Zecconi & Smith, 2000; Janeway et al., 2001). Then, neutrophils are activated and the oxidative burst is initiated. As a consequence, oxygen consumption increase and superoxide (O₂⁻) and hydrogen peroxide (H₂O₂) are produced (Paape et al., 2002). These components are substrate for the formation of hydroxyl radicals and singlet oxygen, which are highly efficient antibacterial substances.

Monocytes/Macrophages

Monocytes constitute about 5% of the total white blood cells of ruminants. After release from the bone marrow, they circulate in the blood stream for 3 to 6 days before migrating into tissues for further differentiation into macrophages (Stabel et al., 1997; Tizard, 2000). Similar to neutrophils, macrophages have phagocytic capacity, but their main function is antigen presentation and cytokine secretion, thereby amplifying the inflammatory response (Politis et al., 1992; Sordillo et al., 1997). After phagocytosis, the pathogens are processed and presented on the cell surface in combination with the major histocompatibility complex class II molecules (MHC-II). These molecules, displayed by APC, are necessary for lymphocyte recognition of antigens. An effective clonal expansion of naïve T-cells

requires the specific T-cell receptor and either CD4⁺, or CD8⁺, co-receptors, which bind to the antigen-MHC complexes and co-stimulatory signals, in the same APC (Janeway et al., 2001).

Dendritic cells

Dendritic cells derive from the same precursor as macrophages, and migrate to peripheral tissues where their role is to survey the local environment for antigens (Janeway et al., 2001). Dendritic cells are recognized as professional APC. In the tissue they have an immature phenotype characterized by low expression of MHC proteins and lack of co-stimulatory molecules (B7). Infections trigger the activation to fully mature dendritic cells with high expression of MHC and B7, and secretion of IL-12, a critical factor for the development of a Th1 immune response (Ismaili et al., 2002; Hope et al., 2003). Differentiation into mature dendritic cells involves the capacity to carry antigens from peripheral tissues to lymphoid organs and activate naïve T-lymphocytes (Janeway et al., 2001).

Lymphocytes

Lymphocytes consist of two different subsets, namely T- and B-cells, which differ in functions and molecule secretions. T-lymphocytes recognize antigens through membrane receptors and are responsible for the regulation of the immune response (Tizard, 2000; Janeway et al., 2001). T-cells are the predominant blood lymphocyte subpopulation in ruminants, accounting for up to 80% of all lymphocytes (Tizard, 2000).

The T-lymphocytes can be subdivided into two main classes, $\alpha\beta$ and $\gamma\delta$ T-cells, depending on the expression of antigenic markers on the cell surface and cytokine production. $\alpha\beta$ T-cells can be further subdivided into both T helper (CD4⁺) and T-cytotoxic/suppressor (CD8⁺) lymphocytes.

CD4⁺ T-cells are activated in response to recognition of antigen-MHC-II complexes and co-stimulatory molecules on APC. As a consequence, activated CD4⁺ cells secrete certain cytokines that can either facilitate a cell mediated (Th1) or a humoral (Th2) immune response (Janeway et al., 2001; Sordillo & Streicher, 2002). Promotion of Th1 immune response is characterized by increased secretion of IL-2 and INF- γ , enhancing cellular responses against intracellular pathogens and viruses, whereas Th2 immune response is characterized by higher production of IL-4, IL-5 and IL-10 supporting humoral immunity (Kehrli et al., 1999). In contrast, CD8⁺ cytotoxic cells have the capacity to kill specific target cells such as tumour cells, or virus-infected cells in combination with the MHC-I associated molecule. On the other hand, CD8⁺ suppressor cells can produce different sets of cytokines such as IL-10 and transforming growth factor beta (TGF- β) with suppressive activity on the immune response (Janeway et al., 2001).

Compared with other species such as human and mice, the ruminant immune system contains a large proportion of $\gamma\delta$ T-lymphocytes. The numbers of $\gamma\delta$ T-cells vary with age and are considerably higher in young animals than in adults, where they constitute 5-10% of the total peripheral blood lymphocytes (Hein & Mackay, 1991). Another characteristic of this cell lineage is their independent recirculation and homing, as $\gamma\delta$ T-cells migrate preferentially to epithelial

surfaces, especially the skin (Mackay et al., 1988). $\gamma\delta$ T-cells have a wide range of functions, including secretion of cytokines such as interferon gamma (IFN- γ), and cytotoxic activity in response to intracellular infections. They may act in the early response to infections, before antigen-specific responses are evident setting a Th1 immune response (Bluestone et al., 1995; Baldwin et al., 2002; Ismaili et al., 2002; Pollock & Welsh, 2002).

The main function of B-lymphocytes is differentiation into plasma cells, which have the capacity to secrete antibodies or immunoglobulins (Ig), but they can also act as APC. As APC they can phagocytose, process and present antigens in combination with the MHC-II molecules to CD4⁺ T-cells, which in turn secrete IL-2 and induce B-cell proliferation and differentiation into either plasma or memory cells (Janeway et al., 2001). Different types of Ig, i.e. IgM, IgG₁, IgG₂, IgA and IgE, are secreted by bovine plasma cells. IgG is by far the most abundant immunoglobulin in blood and plays a major role as opsonin and in antibody dependent cell mediated cytotoxicity (Tizard, 2000; Janeway et al., 2001). IgA operates mainly on epithelial surfaces as a neutralizing antibody preventing bacterial colonization, whereas IgM is the largest molecule and the major immunoglobulin produced during the primary immune response. Its main function is to activate the complement system (Janeway et al., 2001).

Natural Killer Cells

Natural killer cells (NK) are part of the innate immune system. They are large granular lymphocytes with capacities to recognize and destroy tumour and virus-infected cells (Janeway et al., 2001). In contrast to CD8⁺ lymphocytes, NK cells lyse target cells in a non-MHC-restricted fashion (Ljunggren & Kärre, 1990). The mechanism of killing is a process called antibody dependent cell-mediated cytotoxicity (ADCC), where the target cells are recognized through its Fc receptor previously coated with antibodies (Janeway et al., 2001; Moretta et al., 2002).

Changes in the immune system during the periparturient period

In dairy cows, the weeks before and after parturition are a period with high incidence of infections. Diseases may occur when the immune system is unable to respond efficiently to invading pathogens. An efficient immune response relies on the interaction and balance between different cell types and their products. In the periparturient period, large changes occur in hormonal levels and metabolism, adapting the animal to a high metabolism and milk production (Holtenius et al., 1996; Bell & Baumann, 1997; Kehrli et al., 1999). This has consequences for both the innate and adaptive immune responses.

As parturition approaches the total number of WBC increases, mainly as a consequence of higher numbers of neutrophils (Saad et al., 1989; Gilbert et al., 1993). However, the functional capacities of the neutrophils are impaired during this period. One of the key points in the control and eradication of an infection is rapid migration and recruitment of neutrophils to the site of infection (Heyneman et al., 1990; Burton & Erskine, 2003). The first step in neutrophil migration depends on the coordinated function of selectins and β_2 -integrins, i.e. adhesion molecules situated on leukocytes and endothelial cells (Kehrli et al., 1999). As a

consequence, marginating cells stop their rolling and adhere tightly to the endothelium, initiating diapedesis. However, down-regulation and shedding of CD62L molecules from neutrophils have been reported around calving; consequently less numbers of cell are able to migrate into peripheral tissue (Lee & Kehrl, 1998; Paape et al., 2002). In addition, the phagocytic and killing ability of neutrophils are also impaired around parturition (Saad et al., 1989; Hoeben et al., 2000; Mehrzad et al., 2001).

As mentioned, large changes occur during this period also in the adaptive immune response. As parturition approach the proportion of blood lymphocytes and their functional activities, such as cloning expansion and antibody production decrease, reaching a nadir during the days around parturition (Ishikawa et al., 1994; Dettileux et al., 1995; Kimura et al. 1999). The decrease in lymphocyte numbers is due to a net depression in CD4⁺, CD8⁺ and $\gamma\delta$ ⁺ T lymphocytes (Van Kampen & Mallard, 1997; Kimura et al 1999). In addition, the functions of certain subpopulations change. It has been observed that blood CD4⁺ T-cells preferentially produce IL-4 and IL-10 around parturition, while they shift to IFN- γ and IL-2 production during mid to late lactation (Shafer-Weaver et al., 1999). Moreover, CD8⁺ lymphocytes of the suppressor type predominate at this time, which may also contribute to higher levels of IL-4 and IL-10, setting a Th2 or humoral immune response (Shafer-Weaver & Sordillo, 1997). The changes in leukocytes and cytokine production observed around calving result in a suppressed activity of the cellular immune response, which is necessary to deal with intracellular bacteria and viruses, thereby making the animal more susceptible to infections.

Despite the changes detected as parturition approach in the different T-cells subsets, the percentage of B-lymphocytes seems to remain fairly constant (Shafer-Weaver et al., 1996). In contrast, Van Kampen & Mallard (1997) reported a higher proportion of B-cells in blood before and at calving than after parturition. In regards to functional activity of B-cells, a diminished antibody production during the time of parturition has been observed (Nagahata et al., 1992; Dettileux et al., 1995).

Nutrition and health

Energy and protein metabolism

Major nutrients, such as energy and protein, play an important role in the animal's susceptibility to diseases. The energy requirements of dairy cows vary considerably depending on the stage of lactation. The highest energy requirements are detected during the beginning of lactation (NRC, 2001). The recommended energy density of the diet has recently been increased in order to meet the animal's requirements (NRC, 2001). Special consideration is needed to ensure that the large energy requirements of heifers during the prefresh transitional period are met. However, very high energy density diets are not recommended during the dry period as it can have a negative effect on ruminal metabolism with adverse effects on dry matter intake (DMI) (NRC, 2001). The recommended increase in energy density might be warranted as dairy cows often experience a decrease in DMI

before parturition (Van Saun & Sniffen, 1996). A 2 to 4 kg decline in DMI is significant in terms of net nutrient intake.

Protein requirements of the dairy cow are not as clearly defined as those for energy. However, there is a clear difference in crude protein requirements between heifers and mature dairy cows, and between lactating and non-lactating cows. The crude protein requirement for dry and low producing dairy cows is 12-13%, and for high yielding dairy cows it is 15-18% of DMI (NRC, 2001). Heifers need a higher protein intake as they are still growing themselves, but also for the development of the mammary gland. Feeding protein above those recommendations does not confer any advantages (Doepel et al., 2002), or may impair DMI and reproduction, as the capacity to detoxify ammonia is reduced by 40% around parturition (Strang et al., 1998).

Ruminal energy-protein interaction must be addressed in order to optimise ruminal microbial functions. An adequate balance between non-fiber carbohydrates and ruminal degradable proteins optimise production of volatile fatty acids, feed passage rate, and microbial protein production (Hutjens, 1996).

The periparturient period

The peripartum period is the most challenging time for the dairy cows. The mid dry period is considered to be a resting stage between two lactations with low nutrient requirements, but as parturition approaches marked changes in hormonal status to accommodate parturition and lactogenesis occur (Smith et al., 1973; Wettemann, 1980; Bell, 1995). The low requirements of dry cows have given the wrong impression of the critical role of this time. Low nutrient requirements do not mean poor feed quality and management. Generally, a reduction in DMI occurs seven to ten days before calving, but the nutrient demand for the growing foetus and initiation of milk production is increasing at the same time (Grummer, 1995). As a consequence there is a gap in nutrient demand with large changes in nutrient metabolism and metabolic disorders may emerge. A high incidence of metabolic diseases, like ketosis, and infectious diseases, e.g. mastitis and endometritis, occurs in early lactation (Rukkwamsuk et al., 1999; Stabel et al., 2003). Thus, the transitional period needs to be carefully monitored regarding factors such as management, adequate feed composition, e.g. the balance between energy and protein, adequate micronutrient supplementation, and feeding routines to make the transition from the dry to the lactating stage as smooth as possible.

Both over- and under-conditioned dairy cows have a higher incidence of diseases than normally conditioned animals (Rukkwamsuk et al., 1999). Inadequate energy intake during late pregnancy has profound effects on reproductive parameters, such as postpartum interval to first oestrus and pregnancy rate (Randel, 1991). Although overfeeding of dairy cows during the dry period is not advised, overconditioned cows are still observed (Andrews et al., 1991). Fat animals suffer from a more pronounced and more prolonged depression in DMI after calving, resulting in a deeper negative energy balance than cows in normal body condition (Grummer, 1995; Hayrili et al., 2002; Agenäs et al., 2003).

Glucose is an essential nutrient needed for several tissues, and the high demand in the beginning of lactation often exceeds the amount of glucose available (Ropstad et al., 1989; Holtenius & Holtenius, 1996). A four-fold increase in glucose requirements has been reported in high yielding dairy cows at the beginning of lactation compared to nonlactating cows (Bell & Bauman, 1997). The main substrate for the synthesis of glucose is propionate from microbial fermentation of feed carbohydrates. However, especially in early lactation as a consequence of insufficient DMI and high demand of glucose, the cows are also dependent of endogenous substrates, mainly glucogenic amino acids (glutamine, alanine) from degraded endogenous protein sources, and glycerol from adipose tissue mobilization (Bell & Bauman, 1997).

As a consequence of the extensive mobilisation of adipose tissue in early lactation there is a manifold rise in plasma concentration of NEFA (Pullen et al., 1989; Holtenius et al., 2003). The liver plays an important role in fat metabolism, removing NEFA from the blood. In early lactating cows, about 50% of NEFA are oxidised to ketone bodies or reesterified to triglycerides in the liver (Bell, 1995). However, as ruminants do not competently export triglycerides as part of very low density lipoprotein, significant amounts are stored in the liver (Kleppe et al., 1988; Rukkamsuk et al., 1999). Due to hepatic fat deposition, metabolic disturbances such as fatty liver and ketosis emerge with the production of ketone bodies, which can have negative effects on the immune response. A decreased response in mitogen-stimulated T-lymphocytes, and reduced chemotactic capacity, phagocytosis and respiratory burst activities of neutrophils have been reported in ruminants with elevated levels of ketone bodies (Targowski & Klucinski, 1983; Hoeben et al., 1997; Sartorelli et al., 1999; Suriyasathapon et al., 1999).

Micronutrients

Vitamins

Vitamins have been considered to be important dietary components since the early 1900s. Vitamins have diverse functions, and deficiencies can cause a wide range of diseases such as white muscle disease, problems in Ca metabolism, and blood clotting (Horst, 1986; NRC, 2001). Specific nutritional requirements for different vitamins have been associated with changes in the immune response and disease resistance. Therefore, some vitamins are supplemented to bovines to prevent disease, such as mastitis and retained foetal membranes.

Vitamins are divided in fat-soluble (A, D, E and K) and water-soluble (B and C) vitamins. Vitamin K, and the water-soluble vitamins B and C, are synthesised by ruminal and intestinal bacteria, whereas vitamin D is synthesised by ultraviolet radiation of the skin (NRC, 2001). However, vitamins A and E must be provided exclusively from the diet. All vitamins are important for animal health, but here, emphasis is put only on vitamins A and E, as they are considered to play an important role for the immune response.

Vitamin A

Vitamin A, or retinol (the active form of vitamin A), is essential for all vertebrates. Retinol is not found in plants, but its precursor, the carotenoids, do. Among those,

β -carotene has the highest provitamin A biological activity (Bendich, 2004). One IU of vitamin A corresponds to 0.3 μ g of retinol, and 1 mg of β -carotene equals 400 IU of vitamin A (for reference see NRC, 2001). When the animals ingest carotenoids, they are converted to retinol by enzymes in the intestinal mucosa (Chew, 1987; NRC, 2001). In the bovine, large amounts of β -carotene are also absorbed without modifications (NRC, 2001). This might indicate that β -carotene also has an independent function, apart from being a precursor of vitamin A (Bendich, 1993; Bendich, 2004).

The importance of vitamin A and β -carotene in prevention of animal diseases is well documented. Vitamin A is necessary for all cellular division and differentiation (Herdt & Stowe, 1991), and plays a key role in inhibition of keratinisation. Thus, a deficit leads to excessive keratinisation of the secretory epithelia with increased susceptibility to diseases (Reddy & Frey, 1990). β -Carotene, and to a lesser extent vitamin A, have antioxidant activities, quenching free radicals normally produced during cell metabolism (Bendich, 1993). Due to these effects carotenoids have immunomodulatory activities (Michal et al., 1994).

The blood concentrations of vitamin A and β -carotene in dairy cows start to decline approximately 15 days before calving, reaching its nadir at parturition (Johnston & Chew, 1984; Oldham et al., 1991). This drop is mainly due to reduced DMI and, transfer to colostrum (Weiss, 1998). The degree of ruminal degradation of vitamin A varies depending of the diet. High concentrate diets, usually fed during the beginning of lactation, can increase the ruminal vitamin A degradation up to 67% (Rode et al., 1990; Weiss et al., 1995).

Deficiencies in β -carotene and vitamin A around calving have been associated with lower reproductive performance and higher incidence of intramammary infections (Johnston & Chew, 1984; Michal et al. 1994). Tjoelker et al. (1986) found that the phagocytic and killing activities of bovine milk neutrophils were stimulated after in vitro supplementation with retinol and retinoic acid (oxidized form of retinol). In addition, a reduction in milk somatic cell count (SCC) in early lactation was found in cows receiving extra β -carotene or vitamin A (Johnston & Chew, 1984; Chew & Johnston, 1985). However, other reports indicate no effect of vitamin A and β -carotene supplementations during the dry period and early lactation on udder health as measured by SCC (Bindas et al., 1984; Oldham et al., 1991). A possible reason for the variation in response to supplementation might be differences in the vitamin A and β -carotene status at the beginning of the experiments. Jukola et al. (1996) suggested that β -carotene supplementation would have an effect on udder health only when the plasma level of β -carotene is lower than 3.0 mg/l.

Vitamin E

Vitamin E is the generic name of a group of lipid-soluble compounds known as tocopherols and tocotrienols. In nature, they occur under different forms α -, β -, γ - and δ - tocopherols, and α -, β -, γ - and δ -tocotrienols (Kayden & Traber, 1993). α -Tocopherol is the major stereoisomer found in foodstuffs and in blood of cattle, reaching values as high as 95% of total serum vitamin E (Pehrson & Hakkarainen, 1986). Unlike for vitamin A, ruminal activities have no influence on the

metabolism of vitamin E (Weiss et al., 1995). Vitamin E is absorbed in the intestine and transported to the liver in chylomicrons via the lymphatic system. Thereafter only α -tocopherol preferentially appears in plasma, whereas β -, γ - and δ -tocopherol are secreted into bile (Drevon, 1991). This is due to the α -tocopherol transfer protein (α -TTP), which specifically binds the α -form from β -, γ -, and δ -tocopherols, and it has also a further preference for the RRR stereoisomer (Brigelius-Flohé & Traber, 1999). Eight different stereoisomers of α -tocopherol exist, i.e. RRR, RRS, RSS, RSR, SRS, SRR, SSR, and SSS, but the RRR- α -tocopherol isomer has the highest biological activity (Brigelius-Flohé & Traber, 1999; NRC, 2001).

The most common commercially available form of vitamin E for supplementation of farm animals is all-*rac*- α -tocopheryl acetate, which differs in its composition from the natural form, RRR- α -tocopherol (Scherf et al., 1996). All-*rac*- α -tocopheryl consists of a mixture of eight different stereoisomers (RRR, RRS, RSS, RSR, SRS, SRR, SSR, SSS), and it is well established that it exhibits less biological potency than the RRR- α -tocopherol (Brigelius-Flohé & Traber, 1999). As most of the vitamin-mineral mixes for dairy cows contain the synthetic form, this could be another factor influencing the reduced blood level of vitamin E around parturition.

1 IU of vitamin E is equal to 1 mg of all-*rac*- α -tocopheryl acetate, while 1 mg of RRR- α -tocopherol is equal to 1.49 IU of vitamin E (NRC, 2001). These conversion factors are based on studies in laboratory animals, and no peer-reviewed studies have been done to confirm if these values are representative also for ruminants. Results from trials in humans, pigs and beef cattle comparing the effect of administration of natural and synthetic forms of vitamin E, indicate that the bioavailability of RRR- α -tocopherol is roughly twice the bioavailability of synthetic vitamin E (Hidiroglou et al., 1988; Burton et al., 1998; Lauridsen et al., 2002a; Lauridsen et al., 2002b). Emergent evidence from humans and pigs suggests that the natural form of vitamin E is preferentially assimilated by tissues, compared with all-*rac*- α -tocopheryl (Burton et al., 1998; Mahan et al., 2000; Lauridsen et al., 2002a). Based on the NRC dairy cattle requirements for vitamin E, there is still no clear evidence for a suggested amount of RRR- α -tocopherol supplementation. However, the current NRC (2001) requirements for supplementation of vitamin E have increased to 0.8 IU and 1.6 IU/kg of body weight for lactating and dry cows, respectively.

The best understood role of vitamin E in animal health is as biological antioxidant in mammalian cell membranes, providing protection against free radicals (Bendich, 1993; Brigelius-Flohé & Traber, 1999). Vitamin E, as well as Se, have important and complementary roles in prevention of oxidative cell damage. The α -tocopherol molecule is situated in connection with cellular membranes, and attracts polyunsaturated fatty acid (PUFA) molecules with which they form loose chemical complexes until they are metabolised during cell respiration (Putnam & Comben, 1987). The PUFA are unstable and easily attacked by free radicals. α -Tocopherol acts as scavenger of free radicals, preventing more free radicals and hydroperoxide to be produced (Putman & Comben, 1987; Bendich, 1993). Vitamin E also modulates the synthesis of arachidonic acid, and

its metabolites prostaglandins and thromboxanes (McDowell et al., 1996). These arachidonic acid metabolites are important for neutrophil function and amplification of the inflammatory response following pathogen invasion (Smith et al., 1997).

The blood content of vitamin E decreases as parturition approaches and remains low for several days after parturition (Goff & Stabel, 1990; Weiss et al., 1994). Herdt & Stowe (1991) reported that the serum vitamin E concentration depends on the concentration of total serum lipoprotein, which is reduced around calving. However, other factors such as reduced DMI and redistribution of vitamin E to other compartment, such as the udder for colostrum formation, also affect the serum vitamin E content (Goff & Stabel, 1990).

Evidence indicates that vitamin E deficiency around calving may lead to increased incidence of diseases like retained placenta, metritis and mastitis (Smith et al., 1997; Hemingway, 2003). Several reports have documented positive effects of vitamin E on the functions of certain immune cells (Hogan et al., 1990; Politis et al., 1996). Decreased bactericidal activities of blood neutrophils, as well as reduced IL-1 production and MHC-II expression by blood monocytes, were observed around parturition in cows not supplemented with vitamin E (Hogan et al., 1990; Politis et al., 1995). It has been suggested that the serum concentration of α -tocopherol should be at least 3.0-3.5 mg/l to ensure optimal immune cell functions (Weiss, 1998).

Trace elements

Insufficient contents of trace elements in ruminant diets have been related with low disease resistance (Spears, 2000). Several micronutrients such as cobalt (Co), copper (Cu), selenium (Se), and zinc (Zn) have been reported to influence different aspects of the immune system (Paterson & MacPherson, 1990; Reddy & Frey, 1990). However in this presentation, emphasis is put only on Se and Zn.

Selenium

Se is found as a component of different proteins, and recently it was also identified in the enzyme Type I Iodothyronine-5'-deiodinase that converts T_4 to T_3 (Underwood & Suttle, 1999). However, the best-understood Se function is as a part of the enzyme glutathione peroxidase located in the cytosol of the cells (Smith et al., 1997; Underwood & Suttle, 1999). The enzyme catalyses the reduction of hydrogen peroxides. For this reason, Se has a unique role as a cytosolic antioxidant preventing oxidative damages by free radicals (Reddy & Frey, 1990; Underwood & Suttle, 1999). Se is also considered to protect phagocytic cells from autoxidative damage when the respiratory burst is activated. Leakage of free radicals from the phagolysosomes, or failure to detoxify these products, could affect the microbiocidal and metabolic functions of phagocytic cells (Larsen, 1993).

Selenium insufficiency can give serious consequences for the disease resistance of dairy cattle. Health problems such as white muscle disease in calves, and retained foetal membranes, and increased prevalence and severity of mastitis in dairy cows are associated with Se deficiency (Smith et al., 1988; Maddox et al.,

1991; Pehrson, 1993). As mentioned earlier, neutrophils are important components of the early non-specific immune response. Se deficiency has a negative effect on bovine neutrophil functions, such as migration, phagocytosis, intracellular killing and production of chemotactic factors like leukotriene B₄ (Boyne & Arthur, 1979; Aziz et al., 1984, Gyang et al., 1984; Aziz & Klesius, 1986). Moreover, Se deficiency can adversely affect lymphocyte proliferative responses to mitogens in lambs (Turner et al., 1985), and Se supplementation of bovine lymphocytes enhanced IgM production *in vitro* (Stabel et al., 1991). However, high blood Se levels, i.e. above 0.5 mg/l, may have negative side effects on the immune system (Larsen et al., 1988a; Larsen et al., 1988b).

The serum or plasma concentration reflects the current Se status and is more sensitive to short-term changes in the diet (Ullrey, 1987). In contrast, whole blood Se mainly represent the supplementation 30 to 60 days earlier, as Se is incorporated into the glutathione peroxidase in the red blood cells during erythropoiesis (Gerloff, 1992; Underwood & Suttle, 1999).

Important changes in Se status of dairy cows may occur in the peripartum period. The Se concentration in colostrum is four times higher than in milk (Underwood & Suttle, 1999), and Miller et al. (1995) reported a rise in serum Se with increasing days of lactation, probably due to a higher DMI. The recommendation for Se intake in ruminants is 0.1 to 0.3 ppm, but only about 40% of Se administered orally is absorbed (Harrison & Conrad 1984). Moreover, organic sources of Se, such as Se-enriched yeast and feedstuffs with high concentration of Se, usually increase the Se content of the blood and tissues more than inorganic sources (NRC, 2001). The rumen has a strong Se-reducing capacity, and convert oxidized selenite or selenate into elemental Se, which is biologically unavailable (Van Saun, 1990). Diets with high contents of concentrates where nutrients (carbohydrates) are easily fermentable, promote a stronger ruminal reduction of Se (Gerloff, 1992). Moreover, increased contents of sulphur (S) can decrease the digestibility of Se (Ivancic & Weiss, 2001) by reducing ruminal pH, which would favour the conversion of Se from available to unavailable forms, or reducing the Se incorporation into ruminal bacteria (Cummings et al., 1995; Van Ryssen et al., 1998).

Zinc

Zn plays a key role in carbohydrate and nucleic acid metabolism, and protein synthesis as a component of metalloenzymes (NRC, 2001). Zn is also linked with the metabolism of vitamin A, with importance for normal vision (Underwood & Suttle, 1999). Zn deficiency has also been related with reproduction by altered prostaglandin synthesis, which may affect luteal function (Graham, 1991). As a component of the metalloenzyme Cu-Zn superoxide dismutase (Cu-Zn SOD) it can stabilize cell membrane structures, in a similar manner as vitamin E (Reddy & Frey, 1990). Thus, proper immune functions and disease resistance is also related with Zn, and deficiencies can result in atrophy of the thymus and other lymphoid organs (Sordillo & Scott, 1995; Underwood & Suttle, 1999). However, no differences were observed in phagocytosis and killing capacities of neutrophils, or in mitogenic response of blood lymphocytes between calves that had been

supplemented or not supplemented with Zn (Kincaid et al., 1997). In contrast, Fraker et al. (1986) found a depression of T-lymphocyte functions in Zn-deficient animals. In addition, during *Escherichia coli* induced mastitis, the blood concentration of Zn declines suggesting an antibacterial mechanism by which Zn is made less available for bacterial growth (Erskine & Bartlett, 1993). Swedish studies reported no effects on SCC or incidence of mastitis in dairy cows supplemented with Zn, but the milk concentration of IgA, IgG₂ and IgM increased (Hallen Sandgren et al., 1998; Lindmark-Månsson et al., 2000).

Zn is absorbed in the small intestine (Suttle et al., 1982; NRC, 2001), but the absorption is influenced by the Zn content of the diet. Mucosal production of metallothionein limits Zn absorption when the diet content is high and enhances absorption when the content is low (Underwood & Suttle, 1999; NRC, 2001). The chemical composition of the Zn source can also influence its absorption. The absorption is higher in animals supplemented with organic forms of Zn, such as Zn-methionine and Zn-lysine, than in animals supplemented with inorganic sources, like Zn-oxide and Zn-sulphate (Rojas et al., 1995; Kincaid et al., 1997).

As a normal physiologic process, the blood Zn level declines around calving (Goff & Stabel 1990; Xin et al., 1993) due to reduced DMI, transfer of Zn to colostrum and increased stress at this time. Stressors such as parturition and microbial infections decrease the blood Zn concentrations due to redistribution of Zn from blood to tissues, especially the liver (Spears et al., 1991; Underwood & Suttle, 1999). Stress induces liver synthesis of metallothionein, a protein associated with Zn metabolism (Xin et al., 1993).

Aims of the study

The general aim of this thesis was to increase the knowledge about the complex changes in, and interactions between, nutrition and the immune system in dairy cows during the period around calving with emphasis on micronutrients. As parturition approaches several changes occur, both within and around the dairy cow, which may have negative effects on animal homeostasis with adverse consequences for the immune defence and disease resistance.

The specific aims of the thesis were:

- to evaluate changes in blood concentrations of selected vitamins, minerals, and trace elements, as well as changes in leukocyte numbers and functions during the period around calving in dairy cows housed and fed according to Swedish standards.
- to study the effect of feeding intensity during the dry period on blood leukocyte and lymphocyte subpopulations, and neutrophil functions in periparturient dairy cows.
- to evaluate if a blood sample taken during the mid dry period can accurately predict the blood concentration of vitamin A, vitamin E, Se and Zn at calving.
- to compare the effects of supplementation of synthetic or natural vitamin E around calving on α -tocopherol concentration and stereoisomer composition in blood and milk of dairy cows.

Comments on Material and Methods

In this section, the material and methods used in the studies are summarized. A more detailed description is given in each paper.

Location of the Studies

The studies were carried out in Sweden (**Papers I-III**), at the Kungsängen research farm, Swedish University of Agricultural Sciences, Uppsala, and in Denmark (**Paper IV**), at the Research Centre Foulum, Danish Institute of Agricultural Sciences, Tjele.

Animals and Housing

A total of 69 dairy cows of two breeds were used in the studies. All cows in the Swedish studies were of the Swedish Red and White breed (**Papers I-III**), whereas Holstein-Friesian cows were used in the Danish study (**Paper IV**). All studies were carried out during the indoor season from November to April, and the animals were in their second to seventh lactation. The animals were housed in individual tie stalls in both locations (Sweden and Denmark). Ten dairy cows were used in the first study (**Paper I**), twenty-three in the second and third studies (**Papers II-III**) and thirty-six in the fourth study (**Paper IV**).

Experimental Design, Sampling and Diet Composition

In **Paper I**, ten dairy cows were monitored from one month before calving to one month after calving. Jugular blood samples were collected at three time points, one month before, at calving (within 24 hours) and 1 month after calving. The dairy cows were fed 8 kg dry matter (DM) of a total mixed ratio (TMR) composed of 12.5% concentrate and 87.5% grass silage during the dry period. At calving the ration was 11 kg of a TMR containing 36.4% concentrate and 63.6% grass silage. At 1 month after calving the ration was 24 kg of a TMR containing 58.3% concentrate, 4.2% grass hay and 37.5% grass silage. Samples of hay, concentrate and silage were taken once during the experimental period and frozen until analyses of vitamins, minerals and trace elements.

In **Papers II and III**, the animals were randomly allocated to one of three dietary treatments; low (L), medium (M) and high (H) energy rations during the dry period. The groups contained 8, 8 and 7 dairy cows, respectively. All the cows were dried off ten weeks before expected calving and the experimental diets were included eight weeks before parturition was expected. From each cow, EDTA blood for total and differential leukocytes counts was taken from the coccygeal vessel once weekly from 8 weeks before predicted parturition date until 8 weeks postpartum (**Paper II**). Additional jugular blood samples were collected at five time points, i.e. 4-5 weeks and 7-10 days before estimated calving, and 0-3 days, 7-10 days and 4-5 weeks after calving (**Papers II-III**). Composite milk samples were taken from each cow every two weeks during lactation (**Paper II**). In **Papers II and III**, the animals were fed 6 (L), 9 (M) or 14.5 (H) kg of DM of a dry period TMR. The TMR provided 17, 25 and 42 Mcal NE_L/day, respectively, which is 75,

110 and 178%, on average, of the energy requirements for maintenance and pregnancy according to the Swedish feeding recommendation (Spörndly, 1999). From parturition to the end of the experimental period, all cows were fed another TMR *ad libitum*. A detailed description of the composition of the TMR feeds was reported by Agenäs et al. (2003).

In **Paper IV**, the cows were randomly assigned to four groups with nine cows in each group. Vitamin E supplementation was given daily, from three weeks before estimated calving to 14 days after calving as top dressing on the feed. The groups were supplemented with: 1) all-*rac*- α -tocopheryl acetate (SynAc) 1000 IU/day; 2) RRR- α -tocopheryl acetate (NatAc) 1000 IU/day; 3) RRR- α -tocopherol (NatAlc) 1000 IU/day, and 4) no supplementation (Control). Blood samples were taken from the jugular vein 3, 2 and 1 weeks before estimated calving, within 12 hours after calving, and at 3, 7 and 14 days after calving. From each cow two composite colostrum samples were taken within 24 hours after calving and two composite milk samples at 7 and 14 days in milk (DIM). Dry matter intake was recorded and representative feed samples were collected. The animals were fed a TMR according to the Danish recommendations for the dry and lactation periods. The TMR consisted of clover grass silage (28%), corn silage (18%), sugar beet pellets (10%), barley straw (3%) and concentrate (41%). The same TMR was used throughout the experiment but the amount per day differed. The mean amount of energy fed during the dry period until 10 days before expected calving was 21.6 Mcal NE_L/day, and during the close up and early lactation it was 30.3 Mcal NE_L/day. Representative feed samples were taken at the beginning, middle and end of the experimental period for analyses of vitamin E contents.

Cellular Assays

Leukocyte counts

Total and differential leukocyte counts were determined in EDTA blood using a Cell-Dyn^R3500 according to standard procedures at the Department of Clinical Chemistry, Swedish University of Agricultural Sciences, Uppsala, Sweden (**Papers I-II**).

In **Paper II**, determinations of MSCC in composite milk samples were made using a Fossomatic Cell Counter.

Flow cytometric analysis of blood polymorphonuclear leukocytes and lymphocytes

EDTA blood was used for immunostaining of leukocytes, and the proportions of neutrophils expressing CD18 and CD62L, and the mean fluorescent intensity of antibodies to CD18 and CD62L molecules per cell, were measured using flow cytometry (**Paper I**). In **Paper II**, the same technique was used to measure proportions of lymphocytes expressing CD4, CD8, WC1, B-B4, or IL-2R.

The samples were analysed using a FACStarPLUS Flow Cytometry where leukocytes were identified by the expression of CD45. Polymorphonuclear leukocytes and lymphocytes were identified by their size (FSC) and granularity

(SSC), and further evaluation of polymorphonuclear leukocyte adhesion molecules and lymphocyte subpopulations was made by FITC and PE fluorescence (**Papers I-II**).

Polymorphonuclear leukocyte phagocytosis and oxidative burst

Blood was incubated with blue fluorescent beads after pre-treatment with dihydrorhodamine 123. The phagocytosis process was stopped and the erythrocytes were lysed by adding cold tris-buffered ammonium chloride with 0.02% EDTA before flow cytometric analysis. The samples were analysed using a FACStarPLUS Flow Cytometer. The proportion of neutrophils having attached, or ingested, beads was measured by blue fluorescence, while the production of rhodamine 123, due to the activation of the oxidative burst, was measured by green fluorescence (**Paper II**).

Acute phase protein analysis

In **Paper II**, blood without additives was analysed for the acute phase protein serum amyloid A (SAA) using a commercially available ELISA.

Determination of Vitamins, Minerals and Trace Elements

Vitamins

In **Papers I and III**, vitamins A and E were extracted from serum samples with hexan, and the separation was done by High Performance Liquid Chromatography (HPLC).

In **Paper IV**, α -tocopherol in plasma and milk was saponified with KOH, and extracted with heptane. Quantification was done by HPLC. The 2S, RSS, RRS, RRR and RSR blood and milk stereoisomers were also analysed by HPLC.

Minerals

In **Paper I**, the minerals Ca, Mg, Na and K were determined in blood and feed samples using inductively coupled plasma atomic emission spectrometry. Phosphorous (P) in feed samples was analysed using the same procedure, but serum P content was determined by a colorimetric method.

Trace Elements

Plasma Se in **Papers I and III**, erythrocyte Se, and serum Cu in **Paper I**, and serum Zn in **Papers I and III** were analysed using hydrid generation inductively coupled plasma atomic emission spectrometry (ICP-AES).

The trace elements Cu and Zn in feedstuffs were determined according to the description for minerals, and Se according to the blood procedure (**Paper I**).

Statistical Analyses

Analysis of variance was performed on all data using the mixed procedure, and non-significant interactions between treatment and period were excluded from the

models when they appeared to be insignificant in the preliminary analysis (**Papers I-IV**). Probabilities less than 0.05 were considered significant. The fixed effects of treatments, time and interactions are given in more details in each paper (**I-IV**).

In **Paper III**, standard logistic regression was done for each micronutrient to evaluate the relationship between the occurrences of a concentration at two time points.

Results

In this section, the results presented in the studies are summarized. A more detailed description is given in each paper.

Changes in Blood Leukocytes Around Calving (Papers I-II)

Total and differential cell counts

The total white blood cells (WBC) increased significantly as parturition approached compared with before and after calving. This was mainly explained by a significant increase in neutrophil numbers and to a lesser extent in the numbers of monocytes (**Papers I-II**).

The total lymphocyte numbers decreased non-significantly at calving in **Paper I**, and decreased significantly around parturition in relation to the dry period and 4-5 weeks in milk in **Paper II**.

The numbers of eosinophils decreased from its highest before calving to a nadir around parturition and increased again 4-5 weeks in milk (**Paper II**).

Proportions of lymphocyte sub-populations

The proportions of CD4⁺, CD8⁺, B⁺ and IL-2R⁺ lymphocytes were higher after parturition than during the mid dry period (**Paper II**).

Changes in Blood Neutrophil Functions Around Calving (Papers I-II)

Neutrophil adhesion molecules (Paper I)

Ninety-seven percent of the neutrophils were positive for CD18 and CD62L adhesion molecules. The proportion of CD18⁺ molecules was significantly higher ($P < 0.05$) after than before calving, whereas the total proportion of neutrophils positive to CD62L decreased significantly at calving ($P < 0.05$) in relation to both dry and lactating sampling.

Neutrophil phagocytosis and oxidative burst activities (Paper II)

Time had no effect on neutrophil phagocytosis or oxidative burst activities. The mean (SD) proportion of phagocytosing neutrophils was 28.4 (0.4) %, whereas the overall mean proportion of cells with oxidative burst activity was 61.2 (2.2) %.

Effects of Feeding Intensity During the Dry Period on Leukocyte Sub-populations and Neutrophil Functions (Paper II)

Feeding intensity had a significant effect on the WBC and the numbers of lymphocytes, but not on the numbers of neutrophils, eosinophils and monocytes. The WBC was higher in H than in L and M groups, and the numbers of lymphocytes were higher in H than in M cows.

Significant increases in lymphocyte sub-populations were observed after calving relative to the mid dry period in all groups. In L cows, the proportion of B⁺ cells increased, while an increase in CD4⁺ and CD8⁺ cells was found in M cows, and an increase in CD4⁺ and B⁺ cells in H cows. In contrast, the proportion of WC1⁺ lymphocytes decreased after calving in L cows, with a similar trend for the H group.

Feeding intensity had no significant effect on neutrophil phagocytosis or oxidative burst activities.

Changes in Micronutrients Around Calving (Papers I, III, IV)

Vitamins

The serum concentrations of vitamins A and E changed significantly over time. The lowest serum concentrations of both vitamins were observed at calving, compared to all samplings before and after calving (**Papers I, III, IV**). A large proportion of the cows had vitamin A values considered marginal or deficient at calving (**Papers I and III**), while such low values of vitamin E were found in several of the cows both at calving and just before and after calving (**Paper I and III**).

Minerals and electrolytes (Paper I)

The serum level of Ca decreased significantly at calving compared with one month before and after calving. P also decreased significantly at calving, but remained depressed compared with one month before calving. The K level decreased significantly after calving, reaching values under the normal reference range. By contrast, the serum Na concentration increased significantly at calving compared with one month before and one month after calving. On the other hand, Mg remained fairly constant over time.

Trace elements

The serum concentration of Cu was significantly higher at calving and one month after calving compared with the sample before calving (**Paper I**), while serum Zn decreased significantly at calving in relation to one month before and one month after calving (**Papers I-III**).

The concentration of plasma (P-Se), erythrocyte and total Se changed significantly over time. Total Se and P-Se increased significantly at calving in relation to both one month before and one month after calving, whereas erythrocyte Se was significantly higher at calving than one month before (**Paper I**).

In **Paper III**, the P-Se concentration was higher after calving than at and just before calving.

Prediction of Vitamin and Trace Element Status of Periparturient Cows Using Blood Sampling During the Mid Dry Period (Paper III)

The mid dry period concentration of vitamin A did not significantly predict levels considered marginal or deficient (MD) at, or one week after, calving, but it tended to predict levels considered adequate (A) at one week after calving. In contrast, the mid dry period vitamin E concentration predicted the occurrence of MD values at calving, and a similar tendency was observed one week after calving. A vitamin E concentration above 5.4 and 4.4 mg/l one month before calving will, with 90% probability, result in concentrations above MD at calving and one week after calving, respectively.

The mid dry period sample predicted the occurrence of P-Se levels below MD and above A at calving, but no such relationship was observed for Zn. A P-Se concentration above 0.09 mg/l one month before calving will, in 90% of the cows, result in a concentration above MD at calving.

Effect of Natural or Synthetic Vitamin E Supplementation Around Calving (Paper IV)

α -Tocopherol

The blood content of α -tocopherol was significantly higher in the NatAc group than in the NatAlc, SynAc and Control groups. In addition, the concentration was higher in SynAc cows than in Control cows, and a similar trend was observed for NatAlc compared with Control. However, the milk α -tocopherol concentration did not differ between treatments, but varied over time. Substantially higher α -tocopherol concentrations were observed in colostrum compared with in milk.

Stereoisomers

Significantly higher proportions of the 2S, RSS, RRS and RSR stereoisomers of α -tocopherol were detected in the SynAc group compared with the other groups. In contrast, the proportion of the RRR stereoisomer was significantly lower in the SynAc group than in the other groups.

The proportions of the 2S, RSS, RRS, RSR and RRR stereoisomers had the same profile in milk as in blood.

General Discussion

Immune Response Around Calving (Papers I-II)

The total number of WBC and its different cell subpopulations in blood change considerably as parturition approaches in dairy cows. In agreement with other authors (Saad et al., 1989; Lee & Kehrli, 1998; Kimura et al., 1999), we detected a significant increase in the total number of WBC at calving. The changes are explained by a significant increase in the numbers of neutrophils, and to a lesser extent by an increase in monocytes. As parturition approaches large changes in hormonal levels occur in dairy cows, such as an increase in corticosteroids and estrogens, which cause neutrophilia and monocytosis (Smith et al., 1973; Saad & Åström, 1988; Kehrli et al., 1998). The concentrations of other important hormones and metabolites, such as leptin, ketone bodies and NEFA, also change dramatically, which may also affect the leukocytes.

Increased levels of corticosteroids around calving could make dairy cows less resistant to infectious diseases by altering the numbers and functions of leukocytes. Neutrophils are a component of the innate immune system. Therefore they play a unique role as part of the first cellular defence against infections. Despite the presence of high blood numbers of neutrophil in periparturient dairy cows, the incidence of diseases such as mastitis and reproductive problems is high during that period (Eberhart, 1986; Gröhn & Rajala-Schultz, 2000). One explanation is the effect of corticosteroids on the expression of neutrophil adhesion molecules. In our study, we observed a decreased expression of CD62L⁺ neutrophil adhesion molecules around calving, which are indispensable for cell rolling on endothelial cells and further migration of neutrophils into tissues (Kehrli et al., 1999). Down-regulation of selectin molecules means that the marginating pool of neutrophils, rolling along the vessel wall, will shift to the main blood flow stream contributing to the leukocytosis.

Other authors (Saad et al., 1989; Kehrli et al., 1989) have found impaired functional capacities of neutrophils during the period around calving, but we did not detect any significant changes neither in phagocytosis nor in oxidative burst activity. Discrepancies between our findings and other studies may be explained by differences in sampling intervals, methods used, number of cows sampled and/or degree of metabolic disturbances. Moreover, differences in genetic lines between cows could also play a role since not all periparturient cows exhibit immune suppression (Mallard et al., 1998).

Effect of Dry Cow Feeding on Immune Response Around Calving (Paper II)

Several factors such as management, feeding routines and changes in hormonal levels can influence the immune response around calving. Proper nutrition of dairy cows during the dry period is a key factor for their performance in the next lactation. Nutritional mismanagement, such as over- and under-feeding during the dry period predisposes the animals to both metabolic and infectious diseases (Østergaard & Sørensen, 1998; Rukkwamsuk et al., 1999). In our study, the

disease incidence did not differ between groups. This may have been due to the calcium supplementation given to all animals at parturition, which might have influenced the development of periparturient paresis, and possibly also other diseases.

Despite the large differences between groups in the daily energy supply of the dry period diets, limited effects on leukocyte numbers were detected. The numbers of WBC and lymphocytes were highest in the H group, but this was a consistent finding throughout the study indicating a cow effect rather than a treatment effect. However, an increase in the proportion of B-lymphocytes and a decrease in the proportion of WC1⁺ lymphocytes, i.e. $\gamma\delta$ T-cells, in H and L groups were observed after calving. The differences between groups were probably due to changes in lymphocyte proliferation and/or changes in lymphocyte trafficking. It can be speculated that the over- and under-fed cows were under higher metabolic and hormonal stress. Kimura et al. (2002) found lower $\gamma\delta$ T-lymphocytes in metabolically stressed animals with high concentrations of NEFA. In the present study, there was a significant treatment by time interaction in NEFA concentration, and H cows had the highest average NEFA level during the first month post partum (Holtenius et al., 2003). A decreased blood proportion of $\gamma\delta$ T-cells and increased proportion of B-cells in both L and H treatment groups in early lactation indicates an activation of the humoral rather than the cellular immune response. As $\gamma\delta$ T-cells are closely related with the epithelial surfaces (Hein & Mackay, 1991) a decrease in this cell type might indicate a less effective defence against infections (Pollock & Welsh, 2002).

Changes in Micronutrients Around Calving (Papers I, III, IV)

The serum concentrations of vitamin A and E fluctuate considerably during the dry and lactation periods in dairy cows. Higher incidences of infectious diseases have been detected when the serum concentrations of both nutrients are depressed during the period around parturition (Johnston & Chew, 1984; Oldham et al., 1991; Sordillo & Scott, 1995; Smith et al., 1997). In our studies, as demonstrated previously by Goff & Stabel (1990), the serum concentrations of vitamin A and E decreased considerably, reaching a nadir at the time of calving. Moreover, most of the cows reached serum values of vitamin A and E considered marginal, i.e. below 0.2 and 3.0 mg/l, respectively. In many of the cows, low values were also found during the week just before and just after calving. It is probable that a longer time period with marginal blood levels is of more relevance for disease resistance than a short-lasting drop just at calving.

The drop in serum concentrations of vitamins A and E is in response to several factors that occur during the periparturient period. Transfer of the vitamins from blood to colostrum is largely responsible for the observed drop (Goff & Stabel, 1990), as the placenta transfer of those nutrients is very low (Goff & Stabel, 1990; Hidiroglou et al., 1994). Moreover, 7-10 days before calving the DMI is reduced by approximately 30%, and as a result, a reduced uptake of vitamins can be expected (Bertics et al., 1992; Grummer, 1995).

The ruminal activity has also been implicated in the metabolism of vitamin A. As the proportion of concentrates increase, as parturition approaches, the ruminal destruction of vitamin A increases (Weiss et al., 1995). The destruction of vitamin A can be as high as 80% when the level of concentrates in the diet is approximately 70% (Rode et al., 1990).

The variation observed in the blood content of minerals is largely in response to physiological changes that account during the periparturient period in dairy cows. Important minerals such as Ca and P were expected to decrease at calving because of the large demand of colostrum and milk production. In line with other authors (Forar et al., 1982; Underwood & Suttle, 2001) we found an inverse relationship between milk production and plasma P and K concentration.

The trace elements Se and Zn also changed considerably around parturition. Despite that the animals were fed with the similar amounts of Se a disagreement was observed in the periparturient blood Se concentration between **Papers I and III**. In **Paper I**, P-Se increased at calving, followed by a decrease, while in **Paper III**, the P-Se content was higher after calving than at and before calving. The reasons behind the observed differences are unclear, but factors such as numbers of animals, Se content of the feed and, feeding routines may have been responsible. Limited information is available on the effect of high amounts of Se on the immune system, but it is probably wise to avoid overfeeding, as an intake above 3 parts per millions (ppm) of dry matter can have negative side effects on immune functions (Combs & Combs 1986).

Zn, is responsible for normal functions of certain antioxidant enzymes. In our studies, the blood concentration of Zn decreased dramatically at calving mainly in response to colostrum formation (Goff & Stabel, 1990), and to redistribution to other tissues such as, liver. The peripartum in dairy cows is considered a stressful period, and stress can induce synthesis of metallothionein, a protein associated with Zn metabolism, making Zn less available for bacterial growth (Spears et al., 1991; Xin et al., 1993).

Prediction of Micronutrient Deficiency, and Effects of Vitamin E Supplementation Around Calving (Papers III-IV)

The cow's requirements of micronutrients must be provided in the diet. However, the content can vary substantially between feedstuffs, and can be negatively affected by factors like soil type, harvest and storage condition (Herdt & Stowe, 1991; Underwood & Suttle, 2001). Adequate micronutrient supplementation during the dry period could prevent diseases, such as puerperal paresis, and have a positive effect on the immune response. Therefore, to measure the concentration of some micronutrients during the dry period in order to predict the occurrence of deficiency at calving, or in the beginning of lactation, could be of great interest in order to avoid over- or under-feeding of micronutrients. In **Paper III**, we found a positive relationship between the blood concentration in the mid dry period and the occurrence of vitamin E and P-Se levels considered marginal or deficient at calving and in early lactation. The results showed that concentrations of vitamin E and P-Se in the mid dry period above 5.4 mg/l, and 0.09 mg/l, respectively, will

with 90% probability, result in a concentration at calving above marginal levels. These results could be of great importance in order to adjust the micronutrient supplementation in a period with large metabolic changes and a high risk for deficiencies.

The chemical form of the vitamin E supplemented to dairy cows can also influence the changes in serum concentration. In **paper IV**, we showed differences in bioavailability between synthetic and natural forms of vitamin E, measured as serum vitamin concentration. It was clear that supplementation with RRR- α -tocopheryl acetate resulted in higher serum concentrations of α -tocopherol than all-*rac*- α -tocopheryl acetate. Other studies have reported similar findings, for example in beef cattle and pigs (Hidiroglou et al., 1988; Lauridsen et al., 2002a). The results are probably related to the differences in stereoisomer composition of the natural (RRR) and synthetic (RRR, RRS, RSS, RSR, SRR, SSR, SRS, and SSS) forms of vitamin E (Brigelius-Flohé & Traber, 1999), and the fact that α -TTP preferentially incorporates the RRR-isoform. These findings could be of great importance for maintaining a vitamin level in blood above 3.0 to 3.5 mg/l at calving to optimise immune functions. Despite the higher bioavailability of natural vitamin E, the drop in vitamin E around parturition was not avoided and the mean concentration was below 3.0 mg/l at and just after calving. However the results are very promising and open a new field of study, where the level of supplementation and/or the form of vitamin E used has to be studied in more details.

Biological Relevance of the Findings (Papers I-IV)

The results in the present thesis support earlier findings, and open new fields of studies regarding the complex interactions between nutrition and the immune system in periparturient dairy cows.

The suppressed levels of antioxidants, especially vitamins A and E, and the trace element Zn, around calving, reaching values considered marginal in large numbers of cows, suggest that extra supplementation of these nutrients might be needed during this time under Swedish feeding conditions.

The RRR- α -tocopheryl acetate supplementation improved both blood and milk α -tocopherol contents around calving. This observation may be of significant importance with respect to the health of dairy cows, the oxidative stability of milk, and the health and development of the newborn calf.

Data from other studies, suggest that extra vitamin supplementation improve the animal health in the periparturient period. However, such an effect was not confirmed in the present study, most likely due to the small numbers of animals used. To properly evaluate health effects, field studies including large numbers of animals are necessary.

Predicting the need for supplementation of antioxidants during periods of high susceptibility to diseases, such as around calving, may be of both economical and biological importance. A better control of the supplementation of micronutrients may reduce the risk of sub-clinical deficiencies, as well as the risk of toxicity of certain micronutrients, with positive effects on health and productivity.

Finally, the results from the present studies also confirm earlier findings that important processes in neutrophil migration are impaired around parturition, resulting in less efficient neutrophil migration into tissues, increasing the animal's susceptibility to diseases. Moreover, the feeding of the cows during the dry period could further affect leukocyte subpopulations and functions. Over- and under-fed dairy cows suffer larger metabolic changes than normal-fed animals. As a consequence, a Th2-biased immune response predominates, which may have negative consequences for the health of dairy cows around parturition.

Conclusions

- Changes in micronutrients and leukocytes in cows fed according to Swedish standards during the period around calving 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 tissues with negative consequences for the defence against infections. Reduced concentrations of vitamins A and E, and Zn, were also observed at calving, which also can have negative effects on the immune system.
- Feeding dairy cows different nutrient levels during the dry period had only limited effects on numbers of leukocytes, proportions of lymphocyte sub-populations, neutrophil phagocytosis and oxidative burst, and clinical and sub-clinical diseases. However, the data indicate a preference for B⁺ lymphocytes rather than WC1⁺ $\gamma\delta$ T-cells after calving in cows fed high or low energy rations. More studies are needed to clarify the complex interactions between nutrition and immunity during the periparturient period in dairy cattle.
- Analyses of vitamin E and Se, but not of vitamin A and Zn, in blood samples taken from dairy cows during the mid dry period can be used as a tool to evaluate the need for extra supplementation of vitamin E and Se during the periparturient period.
- Daily oral supplementation of dairy cows with RRR- α -tocopheryl acetate in the periparturient period is preferential in terms of increased plasma vitamin E status of cows when compared with supplementation of all-*rac*- α -tocopheryl acetate (synthetic vitamin E) and RRR- α -tocopherol, or no supplementation. Moreover, dairy cows have a preferential uptake of the RRR-stereoisomer of α -tocopherol both in blood and milk. The conversion factor between all-*rac*- α -tocopherol and RRR- α -tocopherol seems to be higher in cows than the official value of 1.36 determined in rats.

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