A Single Prolonged Milking Interval

Effect on Cell Traffic in the Udder and on Milk Composition in Cows

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Uppsala 2007

The present study is a partial fulfilment of the requirements for the Master of Science (MSc) Degree in Veterinary Medicine for International Students at the Swedish University of Agricultural Sciences (SLU), in the field of Animal Reproduction.

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To my family,
with love

Abstract

The aim of the present thesis is to describe the background and specifically investigate the effect of a single prolonged milking interval on cell traffic in the bovine mammary gland and on milk composition. The literature study of the thesis is slightly broader than this and includes also other facts about the bovine mammary gland of interest and relevancy to the narrower subject of the research project. The reason behind performing the research project and writing the thesis is originating in questions raised in relation to practical observations in dairy herds. It has been noticed that after a technical stop in an automated milking system resulting in a prolonged milking interval (PMI), many cows show a short lasting increase in milk somatic cell count (SCC). This can influence the herd milk SCC and may result in reduced payment of the milk due to rules based on that increased milk SCC is associated with lowered milk quality. It is, thus, of economical importance for the producer. By studying the cell traffic in the udder when the SCC is increased but under non-mastitic conditions it was expected that also new information could be gained about the cell traffic in the normal udder which has been poorly studied. The mechanisms behind these SCC peaks and whether they influence milk quality are not clarified.

In the research project 29 dairy cows milked twice daily were included. The cows were exposed to a single PMI of 24 hours by omitting one afternoon milking. Milk samples were taken regularly during 1 week before and 5 days after the PMI and analyzed for SCC, percentage of PMNs, fat, protein, lactose, casein and FFA. The main effects of the PMI were increased SCC and PMN proportion, most pronounced in the milkings during the first day. Interestingly, the proportion of PMNs was of similar size in both milkings day 1 although the SCC in morning milk was much lower than in the afternoon. Usually, the proportion of PMN is known to follow the different SCC in morning and afternoon milking, respectively, well, with lower SCC and proportion of PMN in the morning. The output of number of cells *per hour*, a measure that is not influenced by a possible dilution effect of the large accumulated milk volume in the first morning after the PMI, showed that the highest recruitment rate of total cells and PMN occurred, between the first and second milking after the PMI even if the increase started already during the PMI. After the initial peak, cell counts declined but SCC remained higher while the proportion of PMNs declined to values lower than the baseline value during the rest of the study. Lactose content decreased but in contrast to previous studies, fat and casein increased. The individual cow's lactation stage prior to the PMI had a significant effect on the changes in milk composition. The alterations in milk composition were, however, numerically slight and did not impair the milk quality. The first afternoon milk yield was reduced and, interestingly, remained lower than the baseline value throughout the study.
To conclude: The increased recruitment of PMN shows that there was an enhanced chemotactic activity in the milk already during the PMI without any obvious antigenic challenge. Blood or damaged cells as sources of cytokines is not likely considering the decreased concentrations of serum proteins we observed in the milk and results from previous studies indicating that a single PMI does not cause any cell damage. The results from this study indicate that the PMN infiltration after a single PMI is due to PMN chemotactic factors that are different from the PMN chemotactic factors present in mastitic milk.

Key words: Milking, interval, SCC, PMN, milk, composition

Contents

List of Abbreviations

BSA	Bovine serum albumin
FFA	Free fatty acid
IL	Interleukin
MF	Milking frequency
MFG	Milk fat globule
ODM	Once daily milking
PMN	Polymorphonuclear leukocytes
PMI	Prolonged milking interval
RER	Rough endoplasmatic reticulum
RNA	Ribonucleic acid
SCC	Somatic cell count
TJ	Tight junctions
UDP	Uridine diphosphate

Introduction

Cow's milk is a complex fluid that is a colloidal dispersion of fat globules and protein (casein and whey proteins) in an aqueous solution of lactose, minerals, vitamins and other minor constituents. It is an externally secreted fluid especially designed to nourish the young. From the onset of the life and during the first months of life, milk presents a complete diet with all necessary nutrients. The nutrient value of cow milk in a human diet was recognized long time ago and during the years, increased human consumption and need for milk led to development of a significant dairy sector. The dairy industry has taken huge steps forward with continuous product improvement as well as of cow management. The economy of the modern industrialized dairy sector is dependent on a large and cost-effective milk production and the yield per cow has increased tremendously during the last decades. One of the most important causes to losses in milk yield is subclinical mastitis which is a global problem among dairy cows. The producers' goal to produce more and to lose less has triggered researchers all over the world to try to find ways to diminish the problem of mastitis, and to improve the genetic basis for milk production. In addition, clinical mastitis also includes an animal welfare aspect. As a result of targeted selection, controlled breeding programs, proper nutrition and management, modern cows produce several times more milk than they need for their offspring.

The mastitis reaction, also when less pronounced, leads not only to lower yields but also impaired quality of the milk. Therefore, dairy plants in the western world analyze the milk delivered for the content of mastitis indicators commonly somatic cell concentration. Milk with high somatic cell concentrations often is penalized with a price reduction while extra good quality milk may get premium payment. Stepwise, the upper discrimination value for good milk has become lower and lower, meaning that also moderately increased cell counts play an important role for the producer. In addition to mastitis, the milk cell concentrations are influenced by a number of physiological factors (see further below) and the cell contents have also been found to show a very dynamic pattern over time, with shorter periods of increased values. It is seems that such episodes of elevated cell counts appear without a pathological (mastitis) background. Still the economic consequences for the producer may be the same, reduced milk payments. In the research report that follows at the end of this thesis, the results from a study of short-lasting cell count peaks after a single prolonged milking interval are shown and discussed. However, first some facts about the milk formation and inflammatory reaction of the bovine udder from a literature study are presented

Aims of the Investigation

The aim of the present thesis is to describe the background and specifically investigate the effect of a single prolonged milking interval on cell traffic in the bovine mammary gland and on composition of milk nutrient components.

The specific objectives of the research project were:
- to investigate the pattern of elevated SCC in individual cows after a prolonged milking interval
- to clarify if the increased rate of leukocyte migration is due to an inflammatory reaction by determining the relative numbers of PMNs.
- to examine if the milk nutrient components are influenced in connection with the expected short-lasting SCC peak and
- to find out if the degree of reaction during the SCC peak of individual cows is related to factors including SCC, milk yield, days in milk and lactation number of the cow before entering the study.

Study of Literature

The healthy udder

Physiology of lactation

Galactopoesis includes the onset of production of milk and maintaining a certain level of production throughout the lactation cycle. The initiation of lactation in connection to parturition is a result of interaction of hormones like oestrogen, progesterone and prolactin. Growth hormone, thyroid hormone and insulin are important for maintenance of the milk production. Frequent emptying of the udder is also required for normal galactopoesis. The activity of several hormones expresses a significant influence on milk synthesis and secretion (see e.g. Mepham, 1987; for review, see also Svennersten-Sjaunja & Olsson, 2005).

Oxytocin is a key hormone for milk ejection (see e.g. Mepham, 1987). It is a pituitary hormone synthesized in the hypothalamus and released when the cow sees the calf and in response to teat stimulation. The teat has a well developed sensory innervation. The signals from the stimulated receptors are transmitted to the brain (the supra-optic and paraventricular nuclei of the hypothalamus), thereafter oxytocin is transported to the neurohypophysis from where it is released to the blood circulation and the milk ejection reflex is triggered. The main effect of oxytocin is expressed through its activity on the myoepithelial cells surrounding the alveolei, squeezing the milk into the duct system and further to the udder cistern. It is known that roughly 80% of the milk yield is stored in the alveolar region between milkings. Without proper activity of oxytocin the milk ejection is not working properly.

Prolactin is also a pituitary hormone released after teat stimulation (see e.g. Mepham, 1987). The major effect is expressed by its influence on the metabolism of the epithelial cells through maintaining high concentration of ribonucleic acid (RNA) that is indirectly reflected through higher synthesis of milk proteins. Prolactin can to some extent be replaced by *growth hormone*. However, the main effect of growth hormone is expressed through higher fat synthesis. *Cortisol* has a general supporting effect on the metabolism of lactating animal and additionally supports and aids secretory function of the epithelial cells (see Mepham, 1987).

Milk formation

The complex process of milk synthesis begins in the epithelial cells, the site of accumulation of blood components necessary for milk (Mepham, 1987). The essential nutrients for milk synthesis are in the blood. The precursors of the milk components leave the blood compartment, enter the extracellular fluid and pass the basolateral membrane into the epithelial cells. Some components are transferred from blood to milk unchanged such as some proteins, immunoglobulins and ions. However, most of the essential nutrients are subjected to radical transformation. The mixture of different essential nutrients originating from blood, or directly synthesized by mammary epithelial cells and water, form the milk. The main milk constituents of milk are fat, protein and lactose.

The basolateral membrane is involved in synthesis and absorption of precursors for milk fat. Acetate and b-hydroxibutirate are very important precursors of fatty acid synthesis in mammary cells especially in ruminants, while the glycerol and monoacylglycerides are absorbed at the basolateral membrane and play a significant role in synthesis of fat in other mammals (Jenness, 1986; Mepham, 1987). There are several ways triglycerides can be synthesized in the milk. In the smooth endoplasmic reticulum, triglycerides are synthesized and small fat droplets are formed. The microlipid droplets are released in the cytoplasm with a surface of protein and polar lipids (Mather & Keenan, 1998). Numerous small droplets fuse and move towards the apical part of the membrane where they are being secreted. The fat droplets are wrapped with the epithelial membrane. In this way a milk fat globule (MFG) is formed. The MFG membrane protects the fat from lipolysis. Therefore the amount of membrane material is an important factor for the resistance of MFG to lipolysis (Evers, 2004). To examine the degradation of fat, the content of free fatty acids (FFA) can be measured. The hydrolysis of fat is catalyzed by lipoprotein lipase and it results in higher FFA content (Wiking *et al.*, 2006). The lipoprotein lipase originate from the udder and it is also, somewhat contradictory to its lipolytic effect, involved in milk fat synthesis via effect on the uptake of blood lipids.

The synthesis of proteins goes via amino acids, which are absorbed through the basal membrane of the cell (Jenness, 1986; Mepham, 1987). Several amino acid transport systems are involved in the transport of the different amino acids from one side of the membrane to the other. Inside the cell, amino acids are covalently bound together to form proteins at polysomes (polyribosomes) on the rough endoplasmatic reticulum (RER). From the ribosomes, proteins are transferred

further into the Golgi apparatus and in this way inner and outer proteins are synthesized. The outer proteins are groups of proteins for secretion and consist of several kinds of casein, albumin and lactalbumin. The inner proteins are proteins involved in cell to cell contacts and membrane bound enzymes. Newly synthesized proteins are transferred from RER to the Golgi apparatus where they are processed for transport out of the cell. Casein is a micelle formed in the Golgi apparatus from the casein molecule, calcium and phosphorus. Processing of casein and other proteins, as well as all posttranslational changes, occur in the Golgi apparatus. Milk proteins and lactose are transported to the apical membrane of the cell via secretory vesicles that bud on the Golgi.

Lactose is disaccharide exclusively synthesized in the mammary gland. For the synthesis of lactose two molecules of glucose are required. While one molecule of the glucose is being transported unchanged via highly specified transport system called glucose transporter (GLUT 1) into the Golgy apparatus, the second molecule of glucose is radically transformed already in the cytoplasm (Kuhn et al., 1980; Mepham, 1987). Before entering the Golgy apparatus, glucose is transformed to uridine diphosphate (UDP) glucose) and after to UDP galactose. For the transfer of UDP galactose, active transport is required. Finally, lactose, which is a beta 1-4 covalently bound disaccharide, is synthesised in the Golgy apparatus. As an osmotically active substance, lactose draws water into the cell to balance the intracellular osmotic pressure and represents one milk parameter, minimally subjected to changes under physiological conditions. In balancing the osmolarity in the milk, sodium and chloride ions are important actors. Two enzymatic subunits that have been shown to have a strong impact on lactose synthesis are galactosyltransferase (glycoprotein) and particularly alfa lactalbumin (whey protein). If the availability of lactalbumin is restricted it may be a real limiting factor on the lactose synthesis. Alfa lactalbumin has not a catalytic effect by itself but acts synergistically with galactosyl stranferase. Exclusively in the lactose synthesis, galactosyl transferase has a significant role during the glykoprotein biosynthesis.

The defence of the udder

The protection of the udder from insults and different pathogen invaders is expressed through several mechanisms and functions (Sandholm & Korhonen, 1995; for review see also Sordillo *et al.,* 1997). The first is the teat canal, acting as a physical barrier contributing to protection of mammal gland by its closing mechanism and keratin layer containing antibacterial scleroproteins and fatty acids. It is known that teat canal remains open up to two hours after milking, and in this particular period the role of the keratin and the immune competent cells in Fürstenberg's rosette, located at the inner orifice of the teat canal, is of particular importance. Flushing out microorganisms during milking, is another important defence since it contributes to eliminating bacteria from the udder. It is especially effective against bacteria with a low ability to attach to the tissue, like *Escherichia coli.*

When the invading pathogens have succeeded overcoming the first barrier in the teat canal, there are several kinds of humoral antibacterial proteins which might take part and contribute to bacterial elimination even before the immune response has been triggered. These factors are always present in milk to some extent but their concentrations increase during mastitis reactions. The antibacterial factors may also act in concert with agents in the immune response reaction, thereby enhancing their effect. The most important antibacterial factors are: lactoferrin, transferrin, lysozyme, lactoperoxidase and complement.

Lactoferrin and transferrin are iron-biding proteins (Sordillo *et al.*, 1997). Thus, their antibacterial effect lies in that they compete with the bacteria for iron which is important for the bacteria growth. The main role of lyzosyme is to cause lysis of the bacteria by cleavage of peptidoglycans in the bacteria wall while the lactoperoxidase system acts through oxidation of enzyme structures in the wall of the bacteria. The complement system is more than the other humoral factors mentioned, involved in the immunological reaction by its opsonising ability and effect on chemotaxis.

When the passive defence mechanisms have failed in combating e.g., invading microorganisms, the body must rely on the active immune defence mechanisms as the last line of defence (Sordillo *et al.*, 1997). In contrast to the passive defence, the active immune system needs to be triggered by an antigen, i.e., a microorganism or humoral factor that the body identifies as foreign or as a threat. There are 2 different immunity systems (see e.g. Tizard, 2004a). The first one is the innate immune response, which has the task to act quickly and terminate e.g., an infection before the onset of disease. The inflammatory reaction is the most important innate mechanism. The innate immune system is principally non-specific and is based on the fact that pathogens have characteristics in common that make them chemically very different from normal body components, which enables the body to identify them as foreign. Important in the direct combat of the cause in the innate defence are the phagocytes, especially the fast acting polymorphonuclear leukocytes (PMN). The key aspect of innate immunity is the body's ability to focus these defence mechanisms on sites of microbial invasion. The innate immune system lacks any kind of memory, but is ready to respond immediately when an invading pathogen is identified. (See further under "The inflammatory reaction").

The innate mechanisms are largely non-specific and cannot offer the ultimate solutions to the defence of the body. The system that is capable of recognizing and destroying different antigens, specifically, and that can also learn and remember from the process is the acquired immune system. However, it takes several days or even weeks, before this system becomes active. The acquired immune response consists of two major branches. One branch is directed against extracellular invaders, while the other branch is active against intracellular invaders, such as viruses and protozoa. The extra-cellular invaders are, in principal, destroyed by humoral factors (antibodies), while intracellular invaders are combated by the cell-mediated immune response, mainly lymphocytes of different kinds.

Cells in normal milk

The mammary gland is unique compared to other organs in that leukocytes in fairly large numbers are present also in the normal secretion. Milk from a healthy bovine mammary gland may contain up to 100 000 cells. There appears to be a consensus among mastitis researchers today about this, as an upper threshold value for normal cow milk from a healthy quarter (Hillerton, 1999; Hamann, 2002). However, there are findings indicating that the upper value is even lower like 50 000 cells/ml (Hamann, 2002). The probability of that a mammary gland with milk SCC below 100 000 cells/ml is harbouring an infection is almost negligible (Brolund *et al.*, 1985). The expression "somatic cell count" (SCC) denotes body cells present in bovine milk, which are mainly leukocytes and to a minor proportion, epithelial cells. Milk leukocyte populations consist of neutrophils/polymorphonuclear leucocytes (PMN), monocyte-macrophages and lymphocytes, and to a small extent epithelial cells (for review see e.g. Burvenich *et al.*, 1995 and Sordillo *et al.*, 1997). There are no strict normal values established for the differential cell counts in milk, but most researchers who have studied milk from *healthy* glands, according to the SCC limit mentioned, are reporting values of < 25% PMN and > 70% monocyte-macrophages (Fox & Schultz, 1985; Ostensson *et al.*, 1988; Ostensson, 1993b; Pillai *et al.*, 2001; Rivas *et al.*, 2001; Lindmark-Månsson *et al.*, 2006). Most of the studies show even lower values of < 20% for the proportion of PMN. The contribution of epithelial cells to the milk SCC is low, 1 to 15 % (see e.g. Burvenich *et al.*, 1995).

The milk lymphocytes are mainly T Lymphocytes, being present in percentages up to 60%, and a smaller proportion of B cells of < 20% (Park *et al.*, 1992; Taylor *et al.*, 1994). The T cells consist mostly of CD 8+ cells, known as T cytotoxic cells, and to a minor part of CD4+cells or T helper cells (for review see Sordillo *et al.*, 1997).

In practical mastitis control programmes the upper threshold value for SCC is usually set a bit higher than the 100 000 cells/ml milk that is considered to be the true healthy upper limit. There are several physiological factors influencing the SCC, although to a minor extent (see below). If many cows in a herd would be under influence of such a factor, it might have an impact on the SCC in the tank milk, with elevated values although not due to mastitis. Therefore, it is important set the SCC limit at a reasonably low level so that non-pathological milk for delivery to the dairy plants does not receives complaints.

Physiological factors influencing milk cell counts

Where to put the limit between normal and elevated SCC has been a subject for discussion during the years. The lower the limit, the higher probability that milk with SCC below the limit comes from a mammary gland that is really healthy and free from infection. However, apart from inflammation, SCC is also known to be influenced by several physiological conditions, such as e.g., stage of lactation (Brolund, 1985; Schepers *et al.*, 1997; Piccinini *et al.*, 2007). Increased SCC has been observed after calving for up to two weeks. Thus, shortly after calving,

recorded SCC should be interpreted carefully. McDonald & Anderson (1981) and Miller et al. (1991) reported elevated presence of PMN at the onset and offset of the lactation, while the proportion of lymphocytes was decreased. It is important to emphasize that towards the offset of lactation SCC can significantly be influenced by milk yield, the lower milk yield, the more concentrated and increased SCC (Dohoo & Meek, 1982; Reneau, 1986; Harmon, 1994). Feed and water deprivation are also reflected in a dramatic decrease in milk production and a proportional increase in SCC (Reneau, 1986). Parity may also be reflected in elevated SCC. This can probably be explained by the age and a higher prevalence for mastitis, while parity, per se, most probably has no significant influence (Emanuelson *et al.*, 1988). Season may also affect the SCC under certain conditions. In a study performed in Wisconsin, US (Bodoh *et al.*, 1976) pronounced peaks in SCC was observed during periods of high temperatures in July and August but the SCC was elevated also from April to October, compared with the winter season. It appears that the effect of season is not solely attributable to high temperature. Milk SCC is usually different in different milk fractions. It is higher in foremilk and stripping than in bulk milk, and highest in residual milk (Paape & Tucker, 1966; Ostensson *et al.*, 1988). Finally, milking frequency appears to have a strong influence on milk SCC (Fernando & Spahr, 1983; Stelwagen & Lacy-Hulbert, 1996; Clark *et al.*, 2006) which have been shown to increase in response to prolonged as well as very short milking intervals.

Mastitis

Mastitis means inflammation of the mammary gland and the most common causes to more pronounced mastitis reactions in practice are bacteria and bacterial toxins. However, inflammation can also be triggered by factors released from damaged tissue cells. Mastitis is a protective reaction designed to eliminate infecting agents, neutralize toxins, repair damaged tissue to remove debris at the end of the reaction - and to re-establish normal function of the mammary gland (see e.g. Sandholm, 1995a).

Mastitis is one of the most common disease in dairy cattle in modern dairy production and is causing significant economic losses to the sector. It occurs in two forms differentiated by clinical signs. Clinical mastitis is easy to detect since the symptoms are visible and the udder is harbouring one or several classical signs of inflammation: redness, heat, pain, swelling and/or impaired function, which is seen e.g., as abnormal characteristics of the milk. For the detection of clinical mastitis laboratory diagnostic tools are not required. The main mastitis problem causing the greatest economic losses is, however, the subclinical mastitis, detectable only through laboratory analysis of the milk. The costs are mainly attributable to a lowered milk yield per cow. The reduction of yield may not be very dramatic, but can still be substantial over time because subclinical mastitis is often present for a considerable time and tends to be chronic. This mastitis form may for some producers become a severe herd problem.

Huge efforts have been made for many years to find ways to prevent and cure mastitis through research and improved handling of mastitis problems in practice.

For many decades targeted mastitis control programs with specific and concerted measures have been developed for dairy cows in many countries.

The inflammatory reaction in the udder is not only causing impaired synthesis but also increased concentrations of leukocytes (SCC) in the milk and an altered milk composition that often results in deteriorated quality and processing ability of the milk (Le Roux *et al.*, 2003). Some of the alterations of the milk properties are used for diagnosis of mastitis, in individual cows as well as on a herd level in the control programs and by the dairy plants for quality control of the milk delivered.

The inflammatory reaction

When the pathogen succeeds in overcoming the physical and "natural" barriers, the last line of defence in the udder is triggered; the inflammatory reaction, a part of the innate immune response (sees e.g. Tizard, 2004b; for review see also Sordillo *et al.*, 1997). For initiation of the inflammation the physiologically present macrophages in the milk play a critical role in recognizing the invaders. They are further important producers of cytokines and have also the capacity to sustainable phagocytosis of foreign material like microorganisms although they act late at the end of the inflammatory reaction. The macrophages are also antigen processing cells and important for the antigen presentation to the lymphocytes to initiate antibody production.

The recognition process initiates a pronounced production of cytokines from the macrophages. After the recognition, the first phase of the inflammatory response is characterized by an intensive recruitment of PMNs to the site of the challenge. Thereby, the *proportion* of monocyte-macrophages is decreasing during mastitis. The PMN are attracted to the site of infection by the chemoattractants or cytokines, mainly released by macrophages and lymphocytes but to some extent also from damaged tissue cells. Mainly due to the enhanced PMN recruitment the SCC is dramatically increased at the onset of inflammation, and the proportion of PMN can rise to almost 100% of the total SCC when the reaction is severe (Schalm *et al.*, 1971). In experimental mastitis it has been observed that the early enhanced recruitment and relative presence of PMN in milk is influencing primarily the proportion of monocyte-macrophages while the concentration of milk lymphocytes remains fairly unaltered during this time period (Saad & Ostensson, 1990; Ostensson, 1993a). Among the milk lymphocytes there is a shift towards a predominance of CD4+ T lymphocytes during mastitis in contrast to non-mastitic milk where CD8+ T lymphocytes are prevailing (for review, see Sordillo *et al.*, 1997).

The proinflammatory cytokines represent a group of small soluble proteins expressing a significant role in enhancing all aspects of host defence. The main cytokines observed in the udder are interleukin (IL)1, IL2, IL 6, IL 8, IL 12, tumor necrosis factor (TNF)α, colony stimulating factor (CSF) and interferon (IFN)-γ (for review, see e.g. Sordillo *et al.*, 1997 and Alluwaimi, 2004). The sources of the cytokines are monocytes, PMN, macrophages, lympocytes, endothelial and epithelial cells during the inflammatory process. The role and biological activity of

cytokines is not well examined. The cytokines that have been shown to have a significant effect on activation and recruitment of PMN in the bovine udder are IL1, IL8, TNFα and IFN-γ. CSF is generally not considered to affect cell migration and chemotaxis but some studies have shown that also CSF increases the number of PMN in milk. IL2 appears to have its main effect in stimulating T-cells to express cytokines and IL6 has mainly been shown to affect the transition from influx of PMN to monocytes during the inflammation. The effect of IL12 in the udder has so far been observed just in processes of the acquired immune system.

The phagocytosis function is considered to be the most important defence of the udder and the PMN to be the most important actors to combat mastitis. Pathogens are eliminated through phagocytosis by the PMNs, killed intracellularly by the oxygen burst (for review, see Burvenich *et al.*, 1995). The phagocytic capability of the neutrophils, which have just been recruited to the site of inflammation is greater than that of neutrophils which have been in the milk for some time. This has been ascribed to loss of energy and exhaustion by phagocytosis of casein micelles and fat globules (Paape *et al.*, 2002).

Influence of mastitis on milk composition

The composition of milk is influenced by the health status of the udder. Clinical mastitis results in a pronounced increase of SCC and intracellular enzymes; increase of serum proteins, ions and proteolytic and lypolytic enzymes derived from blood, and a decreased lactose content (see e.g. Sandholm, 1995a). The effect of subclincal and less severe mastitis reactions is usually not as pronounced as in clinical acute mastitis and may not affect all parameters equally strong. The inflammatory reaction is rapidly reflected in elevated SCC which through their proteolytic enzymatic activity, especially by the PMNs, negatively affect the milk quality when they are present in high numbers (Le Roux *et al.*, 2003). Inflammation, additionally, results in decreased yield and altered composition of milk nutrients and milk properties (Auldist *et al.*, 1998) such as decreased lactose and casein content while it results in increase of serum proteins, fat and minor components like minerals and enzymes.

A decreased content of lactose in cow milk with elevated SCC has been observed (Miller *et al.*, 1983). The lactose concentration has been shown to be sensitive to inflammation and a significant decrease has been observed not only during clinical mastitis (Claesson, 1965; Harmon, 1994) but also when the SCC is moderately increased (Berglund *et al.*, 2007). In comparison with other milk components the lactose concentration is very constant, due to its osmoregulating function. The lowered lactose content during mastitis is considered to partly be due to depressed synthesis of lactose but partly also to leakage of lactose from the alveolus to the circulating blood because of disturbed integrity of the tight junctions during inflammation (Stelwagen *et al.*, 1997). To maintain the osmolarity in the milk, sodium and chloride go from the blood to milk resulting in these ions increasing in milk during mastitis.

The total milk protein concentration increases parallel with the SCC (Auldist & Hubble, 1998). Already when the SCC increases to above 100 000 cells/ml the relative contents of the different types of proteins present in the milk are clearly changed (Urech *et al.*, 1999). During inflammation in the udder, total milk protein concentration is increased due to higher content of whey (serum) proteins, while the casein content has been observed to be lowered. According to Korhonen & Kaartinen (1995), major whey proteins such as beta lactglobulin and alfa lactalbumin are negatively affected during mastitis, due to lower synthesis as well as proteolysis but that the elevated content of BSA is due to leakage from the blood into milk through the impaired TJ. The decrease of the casein content is considered to mainly be attributable to epithelial cells damaged during the inflammatory process. The negative balance in the protein content during inflammation can to a certain extent be ascribed the increased content of proteolytic (e.g. plasmin, plasminogen and cathepsin) and lypolytic enzymes that is observed during the course of inflammation. It appears to be a result of leakage through impaired TJ from the blood compartment and leads to enhanced degradation of protein and fat in mastitic milk.

High milk SCC also negatively influences the content of fat in milk apparently due to a decreased fat synthesis in the epithelial cells (Randolph & Erwin1974).

Inflammatory indicators

Total and differential milk leukocyte counts

The inflammatory reaction results in a number of changes in the contents and properties of the milk (see e.g. Sandholm, 1995a) which are used to diagnose subclinical mastitis. For many decades SCC has been the main tool in evaluating udder health and subclinical mastitis and is commonly used in mastitis control programmes. It has been suggested that instead of total SCC, differential cell count, especially the contribution of PMNs to the total milk SCC, should be used. Changes in number of PMNs over time have been found to follow the changes of the SCC during mastitis reactions (Ostensson, 1993a; Sladek *et al.*, 2005) as well as in non-mastitic milk samples from individual cows (Kelly *et al.*, 2000). However, it can be speculated that an increase in SCC could be attributable to increased numbers of other kinds of leukocytes than PMNs as a result of disturbed cell traffic, not necessarily attributable to a common inflammatory stimulus. By analyzing the relative number of PMNs present in milk, it can be established if an inflammatory reaction is really triggered. As in other clinical diagnostic work, differential leukocyte counts in milk are a more precise and sensitive indicator of inflammation in the mammary gland than the total count (Ostensson *et al.*, 1988; Redelman *et al.*, 1988; Kelly *et al.*, 2000). The relative presence of PMNs may be elevated although not enough to be reflected in pathologically increased total number of leukocytes. It has also been reported that, compared to SCC and bacteriology, differential milk cell counts can differentiate non-mastitic, early inflammation and late inflammatory animals (Rivas *et al.*, 2001).

Enzymes

Performing milk cell counts requires fresh milk which is a limiting factor for using cell counts as inflammatory indicator. Analyses of intracellular factors of which each leukocyte can be considered to contain a certain amount, can provide an indirect estimation of the cell content in milk. The milk content of enzymes originating from PMNs increases exponentially with increased milk SCC (see e.g. Sandholm, 1995b). To this group belong N-acetyl-ß-D-glucosaminidase (NAGase), ß-glucuronidase and catalase. NAGase is released during phagocytosis and cell lysis and to some extent from damaged epithelial cells. It is highly correlated with SCC (Emanuelson *et al.,* 1987). NAGase is slightly physiologically increased in the onset and in the end of the lactation. The NAGase analysis, based on a commercial kit of substrate used in an automated microplate assay makes it suitable to use in routine work with large sample quantities.

There are several other enzymes which are increased in milk during mastitis and could possibly be used in detection of mastitis such as myeloperoxidase, different lipases, esterases, phosphatases, and lactate dehydrogenase. Blood proteolytic enzymes such as plasmin degrade casein to fibrin and has an enhanced activity during mastitis (see e.g. Sandholm, 1995b). In theory, plasmin could be used as a mastitis indicator, but the concentration in the milk varies also due to physiological and environmental factors (Politis *et al.,* 1989).

Adenosine triphosphate

Adenosine triphosphate (ATP) is present in all living cells. ATP in milk shows a strong correlation with milk SCC and has also to a minor extent been used as a mastitis indicator in practice (Emanuelson *et al.,* 1987). However, ATP is unstable and rapidly diminishes after the sample is taken if not stabilized by e.g., EDTA.

Serum proteins

Bovine serum albumin (BSA) and antitrypsins have been used as indicators of mastitis (see e.g. Sandholm, 1995b). These parameters indicate increased permeability in endothelium and epithelium as an effect of mastitis. Thus, the content of the blood protein in milk adds information about characteristics of the inflammatory process compared to the SCC. There is not a causal relation between increased permeability and the enhanced recruitment of leukocytes to the milk during the inflammation. The migration of cells is an active process, which is not dependent on high permeability of the capillary wall and mammary epithelium. However, both BSA and antitrypsins have been shown statistically to have a fairly good correlation with the SCC, however not as high as the PMN, NAGase and ATP (Emanuelson *et al.,* 1987). Analyses of serum proteins have mainly been used in research. The advantage of using these parameters in practice, compared to the SCC, is that the samples can be stored frozen

Acute phase proteins

The acute phase proteins (APP) are also increased in the onset of inflammation. They are mainly being synthesized and released by the liver and leak into the milk

from blood (Eckersall *et al.,* 2001). A local production in the mammary gland during mastitis has also been indicated. The APP response starts within a few hours after challenge and usually declines within 24 to 48 h. The most sensitive proteins in cattle are serum amyloid A (SAA), haptoglobin, and alfa 1-acid glycoprotein. While SAA and haptoglobin are substantially increased in the acute phase of inflammation, alfa 1-acid glycoprotein is increased in chronic conditions (Tamura *et al.,* 1989; Eckersall & Conner, 1990). Haptoglobin and SAA have a very high sensitivity and specificity in differentiating between healthy animals and those with mastitis

Lactose

The lactose content in milk is highly correlated with the inflammatory status of the mammary gland. The mastitis reaction causes tissue damage, resulting in disturbed synthesis of milk with depressed biosynthesis of lactose and consequently a lower lactose level in milk (see e.g. Mepham, 1987). In comparison with other parameters lactose is a very constant parameter in milk from healthy udder quarters and appears to be almost constant from one lactation to the next. The relation between SCC and lactose has been a subject of interest (Vangroenweghe *et al.*, 2002; Berglund *et al.,* 2007) with an aim to examine if lactose could be a reliable indicator of mastitis. Analysis of lactose is inexpensive and the handling of milk samples for analysis is easy.

The effect of milking frequency on milk characteristics

The length of the milking interval has been observed to influence the *milk SCC*. Milking once a day increases the SCC (Clark *et al.,* 2006; Stelwagen & Lacy-Hulbert, 1996) and very short intervals have the same effect (Fernando & Spahr, 1983). It has been indicated that once-daily milking on a regular basis also results in increased proportion of PMN along with the increased SCC (Stelwagen & Lacy-Hulbert, 1996) while one omitted milking seems not to influence the proportion of PMN (Fox & Schultz, 1985). High MF has been shown to have a pronounced effect on udder health by increasing mastitis susceptibility (Philpot & Nickerson, 2000).

The length of the milking interval is also known to influence milk composition and yield. Milking cows just once a day appears to result in reduced milk production and changed milk composition (for review, see Davis *et al.,* 1999). Milking frequency (MF) has been shown to be positively correlated with milk yield, while it is negatively reflected through lower content milk fat and protein content (Klei *et al.,* 1997). The influence of increased MF on the fat content can be explained in different ways including increased air exposure due to frequent milking, raised enzymatic activity of fatty acid syntethase, and higher production of short-chain fatty acids (Klei *et al.,* 1997). It has also been observed that increased MF lead to undesirable effects on milk fat, such as increased content of free fatty acids (Svennersten-Sjaunja *et al.,* 2002). The elevated FFA content in milk gives a high risk for off-flavour in the milk. The benefit of increased MF on protein content is in lower activity of the enzyme plasmin, and shorter storage in the udder which lead to lower degradation of protein (Sorensen *et al.,* 2001). Low udder pressure

due to lower milk volume stored in the udder between the milkings when frequent milkings are applied, influences stability of tight junctions and thereby diminish leakage between blood and milk.

Changes in milk characteristics due to a longer milking interval during once daily milking (ODM) have been observed (Stelwagen *et al.*, 1994; Stelwagen & Lacy-Hulbert, 1996). The milking interval should be less than 18 hours to avoid adverse effects on the milk yield and milk quality (Stelwagen *et al.*, 1997). Once daily milking in comparison with two or more daily milkings resulted in significantly higher SCC, protein and fat content in the milk in addition to a decrease in milk volume. It is observed that during ODM mammary cells become leaky, so that movements from milk to blood compartment and vice versa are present to higher degree. The changes in protein content during milking with prolonged intervals have in some studies been shown to be due to increased content of serum protein, suggesting the leakage through the tight junctions (Stelwagen & Lacy-Hulbert, 1996). In the report of Kefford *et al.*, (1995) it was observed that also feed shortage might increase permeability of tight junctions. Protease activity has also been found to be increased in milk from udders exposed to ODM. Higher protein and fat content during ODM was observed by Knutson *et al.*, (1993) and was described as positive energy balance due to the ODM.

In general the casein content is not considered to be affected by the MF. However increased casein content has been reported when applying once-daily milking, regularly (Claesson, 1965; Lacy-Hulbert *et al.*, 1999). This has been ascribed to the large size of the casein micelles, making them un-capable of leaking out through TJs, to the blood compartment.

The lower milk yield observed during ODM, could be ascribed to the decline in the number of secretory cells due to involution. On the other hand there might be a feedback inhibitor effect on the milk synthesis (Hillerton *et al.*, 1990) related to the accumulation of milk during the ODM. Higher daily milk production during increased MF may enhance cell proliferation and cell differentiation.

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Research Report

Effect of a single prolonged milking interval on somatic cell and polymorphonuclear leukocyte counts, and composition of milk in cows

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Abstract

A technical stop in automated milking systems may result in a prolonged milking interval (PMI), after which many cows show a short lasting increase in milk somatic cell count (SCC). The mechanisms behind these SCC peaks are not clarified. Cow composite milk samples were taken at the regular milkings before and during 5 days after a PMI of 24 h, from 29 cows. The milk was analyzed for SCC, percentage of PMNs, fat, protein, lactose, casein and FFA. The main effects of the PMI were increased SCC and proportion of PMN, most pronounced during the first day. Noteworthy is that the proportion of PMNs was of similar size in both milkings day 1 although the SCC in morning milk was much lower than in the afternoon. The average output of number of cells *per hour*, a measure that excludes a possible dilution effect by the larger milk volume at the first morning milking, showed that the highest recruitment rate of total cells and PMN occurred between the first and second milking after the PMI even if the increase started already during the PMI. Lactose content decreased but in contrast to previous studies, fat and casein increased. The alterations in milk composition were, however, numerically slight and did not impair the milk quality. To conclude: The increased recruitment of PMN shows that there was an enhanced chemotactic activity in the milk already during the PMI without any obvious antigen challenge. Blood or damaged cells as sources of cytokines is not likely, considering the decreased concentrations of serum proteins we observed in the milk and results from previous studies indicating that a single PMI does not cause any cell damage. The results from this study indicate that the PMN infiltration after a single PMI is due to PMN chemotactic factors that are different from the PMN chemotactic factors present in mastitic milk.

Introduction

After a technical stop in an automated milking system, many cows have shown a short lasting increase in milk somatic cell count (SCC). The technical stop results

in a prolonged milking interval (PMI), which for many cows may be fairly pronounced. The technical assistance may not be available without some time elapsing and after the stop all cows cannot be immediately milked, but are milked one at a time. The milking interval may be severely prolonged particularly for the cows that had not been milked for several hours before the stop and, additionally, had a long waiting time before their turn to be milked after the stop. Intervals of up to 24 hours have been observed (Pettersson, personal communication 2007).

The mechanisms behind the SCC peaks observed after a single PMI are not clarified. The milk SCC has been found to be highly variable. Besides inflammation, which is the factor with the strongest influence on SCC, several physiological and other factors have been identified to influence SCC such as e.g., stage of lactation, lactation number, milk yield, breed and milk fraction (Brolund, 1985; Ostensson *et al.*, 1988; Schepers *et al.*, 1997; Piccinini *et al.*, 2007) The milk SCC is also influenced by the length of the milking interval. Milking once a day increases the SCC (Stelwagen & Lacy-Hulbert, 1996; Clark *et al.*, 2006); and very short (3 h) intervals have the same effect (Fernando & Spahr, 1983). Additionally there are variations to which the cause is not identified. A relative day-to-day variation in milk SCC for the individual cow of approximately 10% (Sjaunja, 1986) has been demonstrated. The cell traffic in the udder appears to be sensitive to changes in the daily management routines (for review see Ekman, 1998), such as e.g., the length of the milking interval, which, if the changes concern the whole herd, may affect the tank milk with increased SCC and thereby lowering the quality.

The cells in milk are almost exclusively leukocytes. Increased SCC has been found to be associated with an increase in the relative numbers of one particular leukocyte type, the neutrophil/polymorphonuclear leukocyte (PMN); for review see (Sordillo *et al.*, 1997). This relation is observed in individual cow's milk during mastitis as well as when the SCC is influenced by other factors, and further also in herd milk (Blackburn, 1966; Östensson *et al.*, 1988; Östensson, 1993, Kelly *et al.*, 2000). It has been indicated that once-daily milking on a regular basis also results in increased proportion of PMN along with the increased SCC (Stelwagen & Lacy-Hulbert, 1996) while one omitted milking seems not to influence the proportion of PMN (Fox & Schultz, 1985). In milk from healthy, normal udder quarters a majority of the cells are macrophages (for review, see (Burvenich *et al.*, 1995). They have an important role in recognizing invading microorganisms to initiate the defense, the inflammatory reaction. When an inflammatory reaction is initiated by microorganisms or tissue damage, chemoattractants are released and start recruiting humoral factors and PMNs to combat and neutralize the cause. That is the underlying reason why PMNs make the major contribution to the increased SCC in mastitis and can be used as a more sensitive and precise indicator than the total cell count of that an inflammatory reaction is present (Ostensson *et al.*, 1988; Redelman *et al.*, 1988; Kelly *et al.*, 2000).

It can be speculated that an increase in SCC could be attributable to increased numbers of other kind of leukocytes than PMNs as a result of disturbed cell traffic, not necessarily attributable to that a common inflammatory reaction is triggered. It

may also be possible that an increased relative number of PMNs may be present in the milk without the total SCC being elevated. This would indicate an extraordinary leukocyte attraction mechanism. The mammary gland is unique compared to other glands in that high numbers of leukocytes (monocytes/macrophages) are present in its secretion also under healthy conditions (for review see (Burvenich *et al.*, 1995). Regulation of normal cell traffic is not well mapped. Particularly, the short lasting peaks where the SCC returns to normal spontaneously, within a day or even sooner, may be suspected to have a special background. Thus, making differential cell count can provide additional useful information.

In the milk quality control at the dairy, milk SCC has been included as one major important parameter. The reason for this is that increased SCC is associated with changes in the quantity, quality and composition of milk (Auldist *et al.*, 1998). It is most pronounced during clinical mastitis but such changes may be measurable also when the reaction is mild with no clinical symptoms and only modest somatic cell concentration. A lowered milk yield has been observed already when the SCC is moderately increased (Miller *et al.*, 1983). Altered milk composition with decreased relative content of fat, casein and lactose was observed in clinical mastitis (Claesson, 1965; Harmon, 1994). Decreased fat and lactose concentrations have also been shown in moderate and short-lasting periods of increased SCC (Berglund *et al.*, 2007). Besides the described milk alterations related to elevated SCC, the somatic cells have a direct negative effect on milk quality and shelf life (Le Roux *et al.*, 2003).

The length of the milking interval is known to influence milk composition and yield. Milking cows just once a day appears to result in reduced milk production and changed milk composition (for review, see Davis *et al.*, 1999). Fat and protein content increase while lactose decreases in comparison to milking two times per day. The changes in composition seem to be common for both short- and long-term studies. However, it is not fully evaluated how a single omitted milking influences milk quality if the SCC is increased due to PMI as mentioned above.

The characteristics of the kind of milk SCC peaks observed after a stop in voluntary milking systems and the underlying mechanism have not been studied to our knowledge. It could be speculated that the increased SCC after an extended milking interval may be a result of changes in the blood vessels and lymph drainage in the udder tissue due to increased pressure from the large milk volume. It may, however, also be due to inflammatory stimuli from e.g., molecules released from cells damaged by the increased pressure (Stelwagen & Lacy-Hulbert, 1996). It has not been investigated if the various milk constituents are affected during the short-lasting cell peaks. More information about the short-lasting SCC reactions would improve the knowledge about the regular cell traffic in the bovine mammary gland. Additionally, since it may influence the quality of the milk delivered from the farm, it is also a matter of practical concern for the farmer and ought to be clarified.

The aim of the study

The aim of the present study was to investigate the occurrence and pattern of elevated SCC in individual cows after a PMI and to clarify if the increased rate of leukocyte migration is due to an inflammatory reaction by determining the relative numbers of PMNs. An additional aim was to examine if the milk components are influenced in connection with the expected short-lasting SCC peak with this background, and if the degree of reaction regarding milk leukocyte counts and milk composition of individual cows is related to factors including SCC, milk yield, days in milk (DIM) and lactation number of the cow before entering the study.

Materials and methods

Animals

The study was conducted at Kungsängen Research Centre, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden during the month of January. Twenty-nine Swedish Red and White (SRB) cows were included in the experiment. Most of the cows were in mid lactation, 4 cows were in the end of lactation and 1 cow was in the onset of lactation. Lactation numbers varied from 1 to 7 (20 cows were in lactation number 1 to 2). All cows were free from clinical symptoms of mastitis and of other health disturbances before the start of the study. The cows were kept in a tied up system. The feeding management during the winter period includes feeding 4 times daily with silage and concentrate according to the Swedish recommendation. Milking was performed twice daily at 6.30 and 15.30 with a Duovac milking machine system (DeLaval, Tumba, Sweden). The average daily milk yield per cow in the herd according to the Swedish milk recording before the start of the study was 24.8 liter. The study was approved by the Uppsala Local Ethics Committee.

Sampling and experimental design

The duration of the study was in total 12 days during which the cows were exposed to a PMI of 24 h at day 0 by excluding the afternoon milking. Samples of approximately 40 ml of composite cow milk were taken at every milking at day -7, -3, -2, -1, 0, +1, +2, +3, +4 and +5, where minus denotes samples taken before, and plus denotes samples taken after the prolonged interval. Additionally, samples of approximately 80 ml of composite cow milk for analysis of casein and FFA were collected in the afternoon milkings at days -1 and +1 from all cows, and at days +3 and +5 from cows which during day +1 had a SCC that was increased at least 2-fold compared to the afternoon sampling day -1, up to a total SCC value of > 100 × 103 /ml. Each sample was split up in aliquots for the different analyses and stored in a refrigerator until analyzed. Milk yield was measured at each milking by true test equipment.

Milk analyses

Each milk sample was analyzed for SCC, percentage of PMNs, fat, protein, lactose and citrate. Additionally, casein and FFA were measured in afternoon samples collected according to the schedule described (see "Sampling and experimental design"). The SCC was analyzed by fluorescence-based electronic cell count (Fossomatic 5000, A/S N.Foss Electric, Denmark) and PMNs were counted manually in light microscope after staining according to Newman (IDF standard IDF 148-1/ ISO/DIS 13366-1). The content of fat, protein and lactose, respectively, was analyzed by spectroscopic mid infrared technique (MIR; MilcoScan FT 120 A/S N. Foss Electric, Hillerød, Denmark). The samples for casein were stored in a refrigerator in cans with a preservative (bronopole) until analyzed (Arla Foods analysis regulation 2000.004, 200001210). Milk smears were prepared and all analyses were performed within 6 h except for the analysis of FFA when the milk samples were stored for 24 h before analysis. The FFA content was analysed by the Auto analyzer II method (Lindqvist et al., 1975).

Statistical analysis

The data were analyzed using the Mixed procedure with repeated measure ANOVA (Analysis of Variance) in SAS 9.1 (SAS Institute, Cary, NC, USA, 2002). To obtain normal distribution, the data on somatic cell count were transformed to 10 logarithmic values before the analysis. The SP(POW) structure was chosen for both models. The following models were used :

The first model was for observed value of cow i at day t:

$y_{it} = \mu + c_i + \alpha_t + \varepsilon_{it}$

Where μ=overall mean, c_i= random effect of the cow, α_t= effect of sampling day t, ε_{it} = random error. The error ε_{it} and ε_{ijt} corresponding to day t and μ are assumed to follow autoregressive dependence with correlation $\lambda^{/t-\mu/.}$ The covariance structure is accomplished by specified SP(POW) in the SAS program.

The second model was for testing the effect of group i at day t:

$y_{ijt} = \mu + \gamma_i + c_{ij} + \alpha_t + (\gamma\alpha)_{it} + \varepsilon_{ijt}$

Where γ_i = effect of group and $(\gamma\alpha)_{it}$ is the interaction of group and day. The other effects are defined as in model 1.

Before establishing the final statistical model the grouping according milk yield, SCC, days in milk and lactation number were made as a test to motivate model 1.

To establish model 1 all these parameters were checked for influence on milk components.

After examination it was reviled that none of mentioned parameters had any effect on milk cells, and that only days in milk, had a significant effect on milk composition. Thus, the final model included the parameter days in milk. The data are presented as least square means (LSM) with its standard error. After significant F-test ($p<0.05$) least square means were compared in pair wise t-tests at

the 5% level. The baseline value with which post challenge values were compared was calculated as the mean of all pre challenge values for each parameter.

Results

A total of 551 milk samples were collected and analyzed. Results are given in Figures 1–6 and Tables 1–2. Values are statistically compared within morning and afternoon milk, respectively. Of the parameters milk yield, SCC, days in milk and lactation number, only days in milk had a significant effect on the milk characteristics, and only on milk composition.

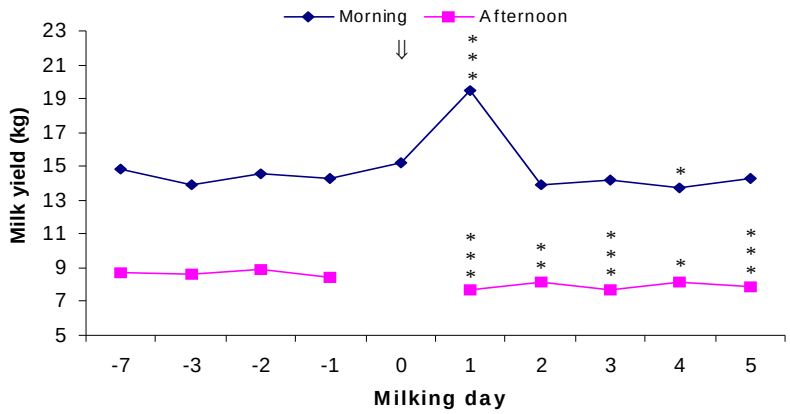

Figure 1. Milk yield in morning and afternoon milking before and after a prolonged milking interval (PMI; arrow) of 24 hours. Data are expressed as LS means. The SE was 0.59 for morning and 0.34 for afternoon milking. Statistically significant differences at each sampling time compared to the base line value before the PMI are indicated by * $p<0.05$, ** $p>0.01$, *** $p<0.001$.Begin entering your text here.

Milk yield

The mean value of milk yield expressed as the baseline value before challenge was 14.6 kg in the morning and 8.6 kg in afternoon, respectively. After the PMI the milk yield was significantly higher in morning milk (19.5 kg) day +1 and significantly lower in afternoon milk (7.7 kg) compared to the baseline, respectively (Figure 1). After the short-lasting peak observed day +1, morning milk yield returned to a level that was similar to the baseline value while afternoon milk yield remained significantly decreased compared to the baseline value, throughout the study.

SCC

The mean somatic cell count per ml expressed as the baseline value before challenge was 21 x 10^3 for morning and 47 x 10^3 for afternoon milking, respectively. After the PMI, SCC increased significantly (Figure 2) in both morning (the first milking after the PMI) and afternoon milking samples, to 27 x 10^3 and 118 x 10^3 respectively. This value represented the highest recorded SCC

value (the peak) in the afternoon milk while the peak in the morning milk was not observed until day +2 and with a notably lower magnitude than the peak in the afternoon milk day +1. After the peaks the SCC in both morning and afternoon milk, respectively, declined but remained significantly higher than the baseline value throughout the study.

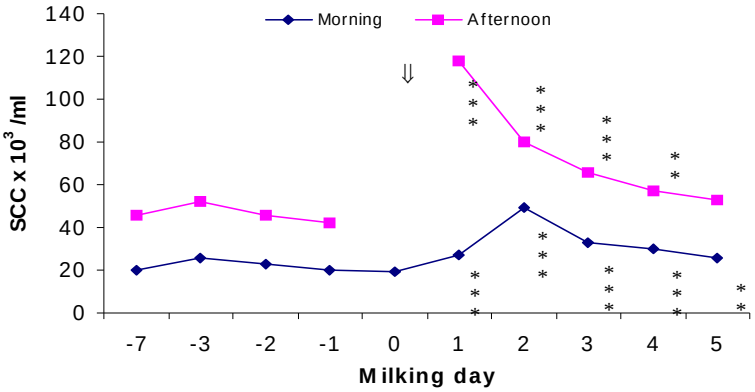

Figure 2. Somatic cell counts (SCC) in morning and afternoon milking before and after a prolonged milking interval (PMI; arrow) of 24 hours. The values given are obtained after antilogarithmic transformation of logarithmic values used in the statistical calculations. Data are expressed as LS means. The SE was 0,07 for morning and 0.06 for afternoon milking, respectively. Statistically significant differences at each sampling time compared to the base line value before the PMI are indicated by * $p<0.05$, ** $p>0.01$, *** $p<0.001$

Since the milk somatic cell concentration per ml is influenced by the milk volume and milk yield differed between the milking occasions in the present study, a more relevant measure to get a picture of the chemotactic activity in the milk at different times during the study may be *cell output, in number per time unit*. When the *output/h* of somatic cells was calculated based on LS-means (data not shown), the output during the PMI was just slightly increased compared to that previously observed in the study, but increased 5-fold day +1 and remained increased but on a lower level also day +2.

PMNs

The mean of the content of PMN per ml expressed as the baseline value before challenge was 15% in morning and 17% in afternoon milk. After the PMI, the percentage of PMN increased significantly day +1 (Figure 3) in both morning (the first milking after PMI) and afternoon milk to 31% and 38%, respectively. It is noteworthy that the PMN peak in both morning and afternoon milk was observed during day +1, in contrast to the SCC in morning milk which did not peak until day +2. After the short and transient peak observed day +1, the relative presence of PMN started to decline day +2 and surprisingly were significantly *lower* than the baseline value from day +3 throughout the study in both morning and afternoon milk.

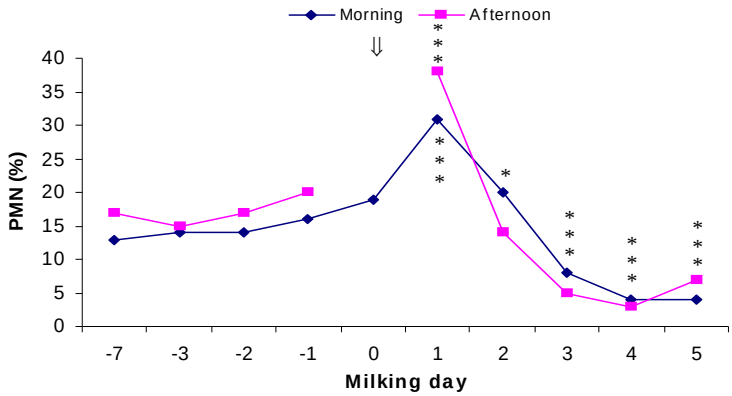

Figure 3. The content of polymorphonuclear leukocytes (PMN, %) in morning and afternoon milking before and after a prolonged milking interval (PMI; arrow) of 24 hours. Data are expressed as LS means. The SE was 2.12 for morning and 2.15 for afternoon milking. Statistically significant differences at each sampling time compared to the base line value before the PMI are indicated by * $p<0.05$, ** $p>0.01$, *** $p<0.001$.

When the *output/h of number* of PMNs was calculated based on LS-means (data not shown) the output during the PMI increased approximately 2-fold, compared to before the PMI, increased further 5-fold during day +1 and remained increased but on a lower level also day +2.

Fat and FFA content

The mean *relative fat* content expressed as baseline value before challenge was 3.8 % for morning and 5.8 % for afternoon milking. After the PMI, the fat percentage increased significantly in both morning and afternoon milking samples, to 4.4 and 7.6, respectively, day +1 (Figure 4). The peak in fat content observed day +1 followed the peak of PMN in both morning and afternoon milk, respectively. After a short transient peak the fat percentage declined and was after morning milking day +2 not significantly changed in either morning or afternoon milk in comparison with the baseline values.

The changes in *total fat yield* (g) *per milking*, (data not shown) after the PMI, followed the same pattern as the fat percentage. The first day after challenge the fat content was significantly increased ($p<0.001$) in both morning and afternoon milking, respectively, compared to the baseline values. The content remained significantly increased ($p<0.05$) also in morning milk day +2 and was occasionally significantly increased ($p<0.01$) day +3 in afternoon milk. The changes followed the patterns of PMN.

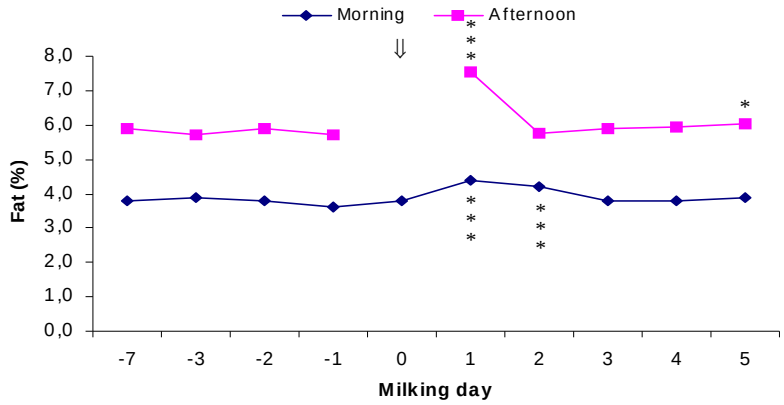

Figure 4. The content of fat (%) in morning and afternoon milking before and after a prolonged milking interval (PMI; arrow) of 24 hours. Data are expressed as LS means. The SE was 0.13 for morning and 0.16 for afternoon milking. Statistically significant differences at each sampling time compared to the base line value before the PMI are indicated by * p<0.05, ** p>0.01, *** p<0.001.

In contrast to all other milk constituents measured, no significant changes in the content of *FFA* were observed after the PMI (Table 1). The value day -1 was set as the baseline value. The numerical drop in FFA (mEkv/l) observed at day +1, day +3 and day +5 was not statistically significantly different compared to the content on day -1. However, when FFA yield was measured relative to the total fat content (mEkv/100g of fat) the decrease from 1.76 before the PMI to 1.33 day +1 was statistically significant. After day +1 FFA was not significantly changed in comparison with the baseline during the rest of the study.

Table 1. The content of free fatty acids (FFA) in afternoon milk expressed as milliequivalents/l (mEkv/l) and yield of FFA milliequivalents/100 g of fat (mEkv/100g) the day before (-1) and day +1,+ 3 and +5 after a prolonged milking interval (PMI) of 24 h. Data are expressed as LS means.

	Sampling day			
	-1 (n=29)	+1 (n=29)	+3 (n=9)	+5 (n=9)
FFA mEqv/l	1.00	0.99	0.98	0.88
FFA mEq/100g of fat	1.76	1.33***	1.69	1.48

Protein, casein and whey protein content

The mean *relative protein* content expressed as the baseline before challenge was 3.4 % for morning and 3.7 % for afternoon milking, respectively. After the PMI, the protein content increased significantly in both morning and afternoon samples

to 3.6% and 3.8%, respectively, day +1 (Figure 5). After the pronounced peak recorded at day +1, values rapidly declined and already in the following day, the protein percentage was not significantly different compared to that before the PMI in either morning or afternoon milk, respectively. However, a significant increase was recorded again from the afternoon milking day +3 and the protein percentage thereafter remained significantly increased in both morning and afternoon milk, respectively, throughout the remainder of the study.

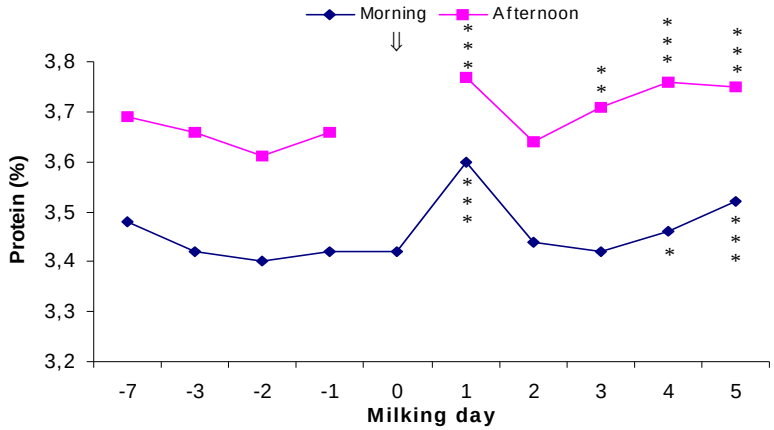

Figure 5. The content of protein (%) in morning and afternoon milking before and after a prolonged milking interval (PMI; arrow) of 24 hours. Data are expressed as LS means. The SE was 0.05 for morning and 0.06 for afternoon milking. Statistically significant differences at each sampling time compared to the base line value before the PMI are indicated by * $p<0.05$, ** $p>0.01$, *** $p<0.001$.

The total protein yield (g) per milking, (data not shown) at day +1 was significantly increased ($p<0.001$) in morning milk while it significantly decreased ($p<0.001$) after the PMI in afternoon milk, compared to the baseline value. After the peak observed in the morning day +1, protein per morning milking declined, starting at day +2, to values that through the rest of the study were not significantly different from the baseline value. However, in afternoon milkings, total protein per milking was significantly lower than the content before the PMI throughout the study except for day +5.

The average *casein* percentage (Table 2) in afternoon milkings was significantly increased to 2.73% ($p<0.01$) and 2.77% ($p<0.05$), day +1 and day +3, respectively, after the PMI, compared to the baseline value of 2.66 % obtained at sampling day -1. At day +5 the casein percentage was not significantly different from the pre challenge value. The total casein output was significantly different at the days +1 and +5, respectively.

The mean whey protein percentage in afternoon milk decreased significantly ($p<0.01$) to 1.04, day +1, compared to the baseline value (obtained at sampling day -1) of 1.08. Thereafter it increased again to values not significantly different from

that before the PMI. The total whey output appears to be non significant, pre challenge versus post challenge values.

Table 2. *The content of casein (%), yield of casein (g), content of whey (%) and yield of whey (g) in afternoon milk the day before (-1) and day +1, +3 and +5 after a prolonged milking interval (PMI) of 24 h. Data are expressed as LS means*

	Sampling day			
	-1 (n=29)	+1 (n=29)	+3 (n=9)	+5 (n=9)
Casein (%)	2.66	2.73**	2.77*	2.74
Casein (g)	221.6	207.7*	225.3	185.58*
Whey (%)	1.08	1.04 **	1.07	1.06
Whey (g)	90.2	79.6	89.8	89.6

Lactose

The mean lactose percentage, expressed as a baseline before challenge was 4.5% for morning and 4.4% for afternoon milk. After the PMI, the lactose content dropped significantly (Figure 6) in both morning and afternoon milk to 4.4% and 4.3%, respectively, at day +1. The lactose content remained significantly decreased compared to the pre challenge value, throughout the study.

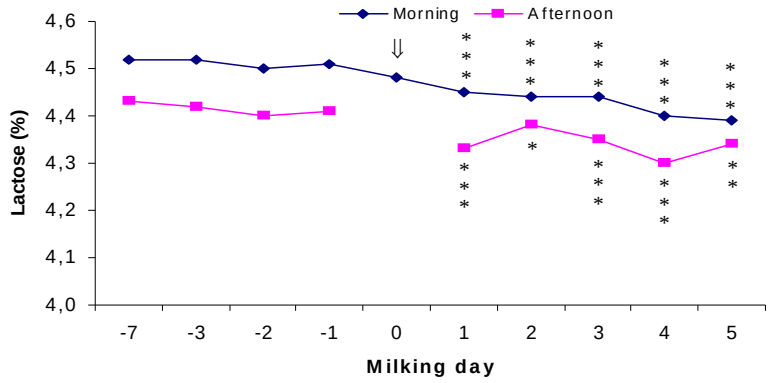

Figure 6. The content of lactose (%) in morning and afternoon milkings before and after a prolonged milking interval (PMI; arrow) of 24 hours. Data are expressed as LS means. The SE was 0.03 for morning and 0.03 for afternoon milking. Statistically significant differences at each sampling time compared to the base line value before the PMI are indicated by * $p<0.05$, ** $p>0.01$, *** $p<0.001$.

The mean total lactose yield (g) *per milking* (data not shown), was at day +1 significantly *increased* ($p<0.001$) in morning milk while it was significantly *decreased* in afternoon milk, compared to the baseline values of morning and afternoon milkings, respectively. After day +1, the total lactose yield in morning

milk dramatically decreased to values that at day +3 and day +5 were not significantly changed compared to the baseline value. In afternoon milk, the reduced total lactose yield remained significantly lower than the baseline value throughout the study

Discussion

The objective of the present study was to examine the influence of a single prolonged milking interval on the milk SCC and proportion of PMNs, and the different milk constituents in cows with a low SCC. The main findings were that one omitted milking resulted in increased SCC and proportion of PMN and that the increase was most pronounced between the first and second milking after the PMI. A notable finding was that the initial change in proportion of PMN in morning milk did not follow the change in SCC in the way that has been known since many decades, that the proportion of PMN is lower in morning milk with usually low SCC than in afternoon milk with usually higher SCC. Interestingly is also that the enhanced infiltration of PMNs to milk occurred without any obvious antigen challenge. These findings indicate a special chemotactic factor behind the enhanced recruitment of PMNs after a single PMI. The alterations in milk composition were numerically slight and did not show any negative influence on the milk quality. The milk yield, SCC, and lactation number of the cows previous to the PMI did not have any over-all effect on the individual cow's reaction in terms of alterations in the different milk characteristics. This is probably attributable to that all cows in the study had low SCC and a good udder health. However, the cow's lactation stage prior to the PMI had a significant effect on the changes in milk composition indicating a different sensitivity of the milk formation process to a PMI, in different stages of lactation.

After the PMI a rise in *SCC* was observed. This finding is in principal in accordance with previous studies (Fox & Schultz, 1985; Stelwagen & Lacy-Hulbert, 1996) but information on milk SCC after a single PMI is scarce. The less pronounced increase in SCC in morning milk at day +1 observed in our study and that the peak value in morning milk, in contrast to the afternoon milk, was not observed until day +2 appears to be a good reflection of the cell output/hr to the milk, which seemed to be nearly un-altered during the PMI but pronouncedly enhanced day +1. The SCC is, in general, known to be lower in morning milk than in afternoon milk (White & Rattray, 1967) which was also observed in our study. It has been ascribed to a different degree of dilution of the cells by the different milk volumes at the two daily milking occasions due to uneven milking intervals. This could partly explain the SCC results also in our study since the milk volume was larger in the morning day +1 compared with the afternoon milking the same day as well as with the previous and subsequent morning milkings. However, according to our observations, the SCC pattern during and after the PMI well reflects the number of cells entering the milk *per time unit*, a measure which exclude the effect of dilution.

Why the peak in SCC and increased recruitment/hr of total somatic cells to the milk, is not observed until the afternoon milking day +1 while increased PMN

percentage in milk is observed already in the morning, remains to be explained. Early increase in the proportion of milk PMN has also been reported by others, however, concomitantly with increased SCC (Stelwagen & Lacy-Hulbert, 1996). Still, in the present study an enhanced PMN migration and activity of PMN chemoattractants in the milk was apparently present already during the PMI and further enhanced during day +1.

Stelwagen *et al.* (1997) described a temporary, reversible disruption of tight junction (TJ) **in**tegrity, due to increased intramammary pressure by milk accumulation after a 24-h milking interval and discussed whether this could facilitate the migration of leukocytes into the mammary gland and thereby contribute to the increased SCC observed in some studies of a single PMI (Fox & Schultz, 1985; Stelwagen & Lacy-Hulbert, 1996). However, leukocytes are not considered to "leak" from the blood but their presence in milk is a result of a targeted recruitment and active migration process. Additionally, we observed decreased concentration of serum proteins (whey proteins) in the milk at day +1, which indicates that the permeability in the capillary walls and mammary epithelium was *not* increased in our study. Thus, enlarged TJs is not a plausible background to the increased SCC and cannot explain the increased relative presence of PMNs observed in our study. The recruitment of PMN was further enhanced during day +1 when the udder was emptied twice, which speaks for that the PMN recruitment was influenced by factors not related to a large milk volume and accumulation of milk, per se.

The increased infiltration of PMN to milk clearly shows that there was an increased chemotactic activity in milk during and after the PMI but without any obvious antigen challenge present. It is also noteworthy that the percentage of *PMNs* in morning milk day +1 did not follow the SCC, in contrast to what has been shown previously in cows milk under various inflammatory and physiological conditions (Blackburn, 1966; Ostensson *et al.*, 1988; Ostensson, 1993; Kelly *et al.*, 2000). These findings indicate a special background to the increased proportion of PMN after a PMI, which is further supported by the extremely rapid return of the proportion of PMN to the baseline level. Manlongat *et al.*, 1998) identified the presence of "physiological" chemotactic factors in mammary secretions influencing the recruitment of PMNs to goat's milk in late lactation and emphasized that increased infiltration of PMNs to the mammary gland under certain circumstances must not necessarily be a result of a pathological process. They also observed different activity of specifically mononuclear leukocyte chemoattractants during the lactation period. Accordingly, our results indicate the presence of "physiological" chemotactic factors in cow milk active in response to a long milking interval. It can be speculated that chemotactic agents may be released from epithelial cells damaged by the increased intramammary pressure caused by the accumulated milk volume after a PMI. However, according to a study by (Stelwagen & Lacy-Hulbert, 1996) no such cell damage was observed even after milking several days with a 24-h milking interval.

Another interesting finding in the present study is that the proportion of PMN decreased to values that were below the baseline value from day +2 in both

morning and afternoon milk, and remained decreased throughout the study. In contrast, the SCC remained increased compared to the baseline value. These results were highly significant even if the changes were numerically modest and indicate a relative decreased attraction of PMNs to the milk, in favour of recruitment of mononuclear leukocytes. This remains to be further explored and explained.

The milk composition was significantly changed the first day after the PMI but the changes in the following days were not consistent except for lactose that decreased throughout the study. The decreased lactose concentration in milk after the PMI in the present study needs further discussion. Lactose is the key for osmotic regulation in the udder and a drop in lactose content is often accompanied by a drop in milk yield. There are several probable explanations to reduced lactose content in milk. Either it could be ascribed to leakage out of lactose from the milk through impaired TJs or it could be attributed to lower synthesis of lactose during the PMI. Since the unchanged content of serum proteins in milk speaks for an unchanged integrity of the TJs, a lower synthesis is more likely. Since lactose plays a key role in regulating the osmotic pressure it is also influenced by the content of ions in milk. More detailed studies would be necessary for further understanding.

The elevated *protein content* was shown to be due to an increase in *casein* while the serum proteins (whey proteins), although with slight numerical changes, remained statistically unchanged. Increased casein content has been reported from previous studies of PMIs when applying once-daily milking, regularly. Claesson (1965) observed a higher concentration of casein during once-daily than twice-daily milking as did also (Lacy-Hulbert *et al.*, 1999). The increase has been explained by the large size of the casein micelles, making them un-capable of leaking out through TJs, to the blood compartment like other milk constituents may do. It ought to be emphasized that these studies concern the long term effect of once-daily milking and the reason behind might not be a plausible explanation to the increase observed in our study, of a single PMI. The increased proportion of casein observed in our study was probably attributable to a concentration effect by the alterations in milk yield at day +1. Further, the increased casein content observed while whey protein content remained un-changed, indicates no increased presence of plasma proteolytic enzymes in the milk and thus unchanged permeability of endothelium and epithelium.

The *fat* content was significantly increased after PMI. The changes in fat might at least partially be ascribed to a concentration effect. FFA is undesirable in milk due to its degradation of fat and rancid flavor. Accumulation of FFA in the milk is related to higher hydrolysis of triglycerides catalyzed by lipoprotein lipase. It is known from previous studies that increased milking frequency may result in elevated FFA content in milk (Klei *et al.*, 1997; Wiking *et al.*, 2006). In the current study, the level of FFA remained statistically unchanged after the PMI, although it was numerically slightly decreased. Since fat content increased while the FFA remained unchanged, apparently, there was no effect of lipoprotein lipase, originating from blood plasma. This further supports that the TJs kept their integrity during the study.

As expected, the PMI resulted in significantly elevated milk *yield* at the first milking in the morning of day +1 due to accumulation of milk in the udder during the PMI. In the rest of the study the morning milk yield was not changed compared to before the PMI. In contrast to the morning milk, the afternoon milk yield day +1 was reduced and, remarkably, remained significantly lower than the baseline value throughout the study. A probable mechanism behind that afternoon yield particularly was reduced is difficult to identify. There is little information available of the effect of one single PMI on the milk yield on a whole, but (Fox & Schultz, 1985) reported decreased milk yield after a single PMI. A considerable reduction of milk yield was also observed by Claesson *et al.* (1959) where one milking per week was omitted. Most reports concern the effect of once daily milking when applied during a period of time. In several such studies a lowered milk yield has been observed in cows when milking was reduced to once per day (Davis *et al.*, 1999; Lacy-Hulbert *et al.*, 1999; O'Brien *et al.*, 2002). However, these cows were regularly exposed to a PMI and the results show the long term effect of less frequent milking and regularly higher intramammary pressure. The suggested mechanisms behind the reduced milk yield observed in the studies referred to may therefore not explain the short term results seen after a single PMI as in the present study. However, since the lactose content decreased significantly it appears likely that the drop in yield could be due to a negative influence of high intramammary pressure on the milk secreting cells. However, Stelwagen and Lacy-Hulbert (1996) showed that a single PMI did not cause any damage to the mammary secretory epithelium. Additionally, a lingering effect of the increased pressure during the PMI over several days while the milking continued twice per day, seems not to be a plausible explanation for the lowered milk yield. It still remains to be explained.

To summarize: The results from the present study indicate a special chemotactic background to the increased proportion of PMN in milk observed the first days after a PMI. The increased recruitment of PMN shows that there was an enhanced chemotactic activity in the milk already during the PMI without any obvious antigenic challenge. Blood or damaged cells as sources of cytokines is not likely considering the decreased concentrations of serum proteins observed in the milk and results from previous studies indicating that a single PMI does not cause any cell damage. Thus, the PMN infiltration after a single PMI seems to be due to PMN chemotactic factors that are different from the PMN chemotactic factors present in mastitic milk. After 2 days the proportion of PMN decreased and remained lower than the baseline throughout the study while the milk SCC remained higher than the baseline value. Milk composition was not remarkably changed after the PMI and it does not influence milk quality. The alteration in most milk constituents excluding lactose, appears to be attributable mainly to concentration effects due to the changes of the milk yield. At the first morning milking after the PMI, the yield was higher than at other milkings during the study due to accumulation of milk while the first afternoon’s milk yield was reduced and, interestingly, remained decreased throughout the study. The elevated milk protein content was due to an increase in casein, while the whey proteins remained unchanged. Several findings support that the TJs were apparently not affected by the PMI in this study.

Acknowledgements

This study was funded by SLF (Swedish Farmers Foundation for Agricultural Research).

The authors want to express their sincere thanks to Lennart Norell, Unit of Applied Statistics and Mathematics, Swedish University of Agricultural Sciences, Uppsala for excellent statistical advice.

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Acknowledgements

This study was carried out at the Department of Clinical Sciences, Division of Reproduction, Faculty of Veterinary Medicine and Animal Science, Sweden, University of Agricultural Sciences, Uppsala, Sweden.

I gratefully acknowledge scholarship from Swedish Institute without which it was impossible to do this study.

Prof. Björn Ekestern, Head of the Department of Clinical Sciences, I convey my deepest gratitude and regards for offering me a great opportunity to work on my study and allowing me to use the Department's facilities.

Associate Prof. Lennart Söderquist, Head of Division of Reproduction, I convey my gratitude and regards for providing me all sorts of facilities to carry out my research in your division. Many times you were a good friend helping with difficulties in Swedish terms, translating them for me.

Associate Prof. Karin Östensson,, my Scientific Adviser, you have helped me during whole period of studying. It has taken a lot of patience to show me many tips, good ways of thinking and directions. Without you I would never found my way out from the very first idea in the study and raw data. Writing a paper and thesis would never be done without touch of your "magical pen".

I convey my thanks and regards to Prof Lennart Norell for his excellent statistical assistance. Without you I could only stay captive with numbers forever, with no results.

Marie Sundberg, our best guardian for life in Sweden. With out of all that assistance it would be far more difficult. Sometimes I was dreadful providing too many questions, but you have had always few minutes and a smile for all of us.

I wish to thank to my dear friends, students from Division Jatesada , Fernando, Paweena, Fikre, and Yoseph for helping me all the time during my studies. Without them and their unselfish help in solving problems with computers and statistics, this studying would be "mission impossible". To my office friend and sister Bojana. I wish to thank her for unselfish support during all studies and especially in a moments, when her help was more than welcome. Once more in my life I was LAKI (lucky) to share office with someone endlessly interesting, cheer and generous like Bojana is.

For all the stuff in the Division, I convey my thanks to give me opportunity to become a "part of the family" and enjoy many nice hours and happenings, and to understand a better life style and habitude in Sweden.

Thanks to my dear parents and my brother Nenad for supporting me endlessly, via internet supplying me with the for carrying about my son all this time and touching me with love with many packages' and letters of encouragement.

Great thanks to my relatives in Uppsala, aunt Stojka and uncle Boris for taking care of me from the early beginning living in Sweden.