Respiratory burst activity and viability of bovine blood and milk neutrophils during different stages of lactation and mastitis

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by

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Cover:

The illustration on the cover represents the defense of one of the many precious Iran’s ancient buildings (~550 B.C). Since millennia ago, Iranians have uninterruptedly been defending Iran soil, culture and civilization, as neutrophils regularly do for mammary gland against pathogens.

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To the soul of my parents Ameneh and Yousof, who did everything for their children; to my wife Firouzeh and my son Behnam, who are my everything
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December, 2002 Merelbeke
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List of abbreviations

Ab: antibody
ANOVA: analysis of variance, the statistical approach of data analysis
AUC: area under the curve
BDHC: benzidin dihydrochloride
bST: bovine somatotropin
CD: cluster of differentiation
C5: fifth component
cfu: colony forming unit
CL: chemiluminescence
CLI: chemiluminescence index
CV: coefficient of variation
DMSO: dimethyl sulphoxide
DPBS: Dulbecco’s phosphate buffered saline
E. coli: Escherichia coli
e: experimental error term
FCM: Flow cytometry
FS: forward scatter
HR: heart rate
h²: heritabilities
i.m.: intramuscular
i.mam.: intramammary
i.v.: intravenous
IFN: interferon
IL: interleukin
IMI: i.mam. infection
L: lymphocyte
LBP: lipopolysaccharide binding protein
LPS: lipopolysaccharide
LSD: least significant differences, the statistical approach of data analysis
M: macrophage
mA: milliamper
mAb: monoclonal antibody
mM: milli mole
MPO: myeloperoxidase
NADPH: reduced form of nicotinamide adenine dinucleotide
O²⁻: superoxide anion
OH⁺: hydroxyl radical
P: probability
PBS: phosphate buffered saline
PI: propidium iodide
PCH: post-challenge hour
PIH: post-infection hour
PK-C: protein kinase-C
PMA: phorbol-12-myristate-13-acetate
PMN: polymorphonuclear leukocyte
R: a statistical term of coefficient of determination
RLU/s: relative light unit per second; a measurement unit for chemiluminescence
ROS: reactive oxygen species
RR: respiration rate
RT: rectal temperature
*S. aureus*: *Staphylococcus aureus*
*S. uberis*: *Streptococcus uberis*
SCC: somatic cell count
SD: standard deviation
SEM: standard error of the mean
SOD: superoxide dismutase
SS: side scatter
TNF-α: tumor necrosis factor alpha
µ: statistical factor for mean of measurement
WBC: white blood cell
Y: dependent variable for statistical analyses
Preface
Preface

This Ph.D. thesis comprises several research papers of which Jalil Mehrzad is the first author. Each paper addresses one thing in common: blood and milk polymorphonuclear leukocytes (PMN) functions in high yielding dairy cows. Pathophysiology of mastitis links to parturition; maximal mastitis incidence appears during early lactation. Both mastitis incidence and pathophysiology of mastitis associate with “PMN dysfunction”. This has led us to conduct some fundamental research on blood and milk PMN reactive oxygen species (ROS) production, viability and maturity during several physiological and mastitis conditions.

The general introduction is an overview on the PMN function and its role on mammary gland defense against pathogens. Several PMN functions are discussed. Potential physiological and pathological influencing factors on PMN ROS production and viability are also addressed.

The first part describes the development and evaluation of methods. This part consists of three chapters. In chapter 1 the issue of proper sampling and isolation procedure of blood and milk PMN is examined. In chapter 2 and 3, a detailed description of the optimized measurement conditions of viability and chemiluminescence (CL; the most accurate approach for PMN ROS quantification) technique for bovine blood and milk PMN is presented.

The experimental part is divided in three sections. The first section is study on healthy cows. This comprises two chapters; first chapter focuses influence of lactation cycle on blood and milk PMN CL and viability; second chapter addresses contribution of lactation number to blood and milk PMN CL and viability. The second section is the experiments performed during mastitis, comprising two chapters; first chapter deals with endotoxin mastitis and next chapter focuses on E. coli mastitis. The impact of these two pathogens on blood and milk (inflamed and non-inflamed quarters) PMN CL and viability is explicitly discussed. The last section of the experimental part is the study on susceptibility of E. coli mastitis. In this section the crucial role of blood and milk PMN CL on cow-E. coli interactions and the severity risk is exclusively examined.

This thesis would make the complex pathophysiology and immunology of early lactation-related infectious diseases a little bit more comprehensible.

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Jalil Mehrzad
This introduction is based on:


Abstract

Defense of the mammary gland against mastitis-causing pathogens is mediated by many cellular and acellular factors. The professional phagocytic cells of bovine mammary gland, polymorphonuclear neutrophils (PMN) and macrophages, comprise the first line of immune defense against invading mastitis pathogens. PMN are the only leukocytes in milk compartment that are capable of producing large amounts of reactive oxygen species (ROS) to kill phagocytosed bacteria. In this introduction, the role of PMN function as an effective defense against intramammary pathogens in dairy cows and (patho)physiological influencing factors on blood and milk PMN ROS load and viability is discussed. Apart from playing crucial role in the first line of immune defense mechanism, the PMN can also interfere with the complex interactions of second line of defense against pathogens. Chemiluminescence (CL) of PMN is widely used tool of mimicking cow’s first line of immune defense mechanisms. To minimize mammary tissue damage caused by bacterial toxins and oxidative products released by PMN, elimination of invading bacteria should proceed quickly. This can provide balance between inflammation reactions, bactericidal activity and tissue damage. Post-diapdetic PMN viability (as an index of PMN quality in tissue) might offer such a balance. The well balance between host-pathogen interactions might be affected by physiological (e.g., stage and number of lactation) and pathological (e.g., local-systemic effect of mastitis) status of dairy cows. Hormones, metabolites and acute phase proteins also influence on PMN ROS production and viability, thereby affecting on the outcome of mastitis. This is especially the case around parturition. PMN function in healthy cows after parturition is highly heritable and has been related to the cow’s susceptibility to clinical mastitis. Despite sensible antibiotic application and advances in molecular biology and nutrition, the proper immunomodulation in high yielding dairy cows against mastitis is still far from assured. The most prominent ROS, hypochlorous acid or HOCl, is produced by myeloperoxidase (MPO)-hydrogen peroxide (H₂O₂)-halide system. The long-term and fundamental solution for early lactation-related infectious diseases is to strengthen their first line of immune defense mechanism by means of attainable physio-immunological approaches. This requires a comprehensive study on cellular immunity throughout lactation and during mastitis.
1. Introduction

Polymorphonuclear neutrophils (PMN) are the primary mobile phagocytes of the immune system. Their importance for non-specific defense of mammary gland has long been a crucial concern in high yielding dairy cows (Burvenich et al., 1994; Paape et al., 1996; Kehrli et al., 2001). Clearly, PMN chemotaxis, diapedesis, phagocytosis, and eventually microbicidal activity each contributes to the ability of PMN to provide an effective first line of immune defense for the mammary gland. Any condition that depresses PMN functions adversely affects the udder’s resistance to invasive infections. The PMN reactive oxygen species (ROS) production can be quantified following stimulation with soluble agents or with particles. The most widely used technique to quantify PMN ROS production is chemiluminescence (CL) (Allen et al., 1972; Weber et al., 1983; Piccinini et al., 1999; Mehrzad et al., 2000a). As phagocytosis-induced and/or non-induced CL reflects intracellular and extracellular oxidation-reduction reactions (Webb et al., 1974; Babior, 1984), changes might offer some evidence about the cow’s susceptibility to early lactation-related infections. The mammary gland is an extremely important organ from an economical, immunological and nutritional point of view and mastitis is one of the crucial lactation-related diseases (Blowey, 1984; Burvenich et al., 1994; Kehrli et al., 2001). Impairment of PMN, originating from the bone marrow, is a peculiar feature during the periparturient period (Burvenich et al., 1994; Hoeben et al., 2000a). This impairment might be cumulative upon influx into the milk. Generalised PMN impairment can be multifactorial, e.g. due to metabolic (Suriyasathaporn et al., 1999) and hormonal (Gray et al., 1982; Kremer et al., 1993; Persson et al., 1993; Suriyasathaporn et al., 2000) changes. Another crucial aspect of mastitis is the viability of PMN, which is apparently influenced by the pathological conditions of the gland (Piccinini et al., 1999). However, little study is available on PMN viability.

Several antimicrobial systems exist in the mammary gland (Burvenich et al., 1994; Paape et al., 1996; Kehrli et al., 2001). Nevertheless, the presence of PMN in milk provides a central natural defense for the gland (Burvenich et al., 1994). Under both clinical and experimental conditions, mastitis cows show a large variability in illness and a wide range of pathological responses (Heyneman et al., 1990; Vandeputte-Van Messom et al., 1993; van Werven et al., 1997). Still to be answered is the key question: are there considerable variation in blood and milk PMN CL, viability and number during physiological and pathological conditions of the mammary gland? In addition to a sustainably constant hematopoeigenesis and PMN recruitment into the milk during physiological and mastitis conditions, it is still inconclusive that mammary gland PMN CL can make a huge difference in response to invading pathogens.

2. Ultrastructure of bovine PMN

The fine structure of bovine PMN has been exclusively demonstrated in classic studies (Paape and Wergin 1977a; b; Paape et al., 1979; Paape et al., 2002). The cell is delineated by a plasma membrane that has a number of functionally important receptors. These include L-selectin and β2-integrin adhesion molecules associated with the binding of PMN to endothelial cells that are important for migration into sites of infection (Kishimoto et al., 1989; Zimmerman, 1992). Membrane receptors for the Fc component of the IgG and IgM classes of immunoglobulins and complement (C) C3b are necessary for mediating phagocytosis of invading bacteria (Paape et al., 1991). Dying or apoptotic PMN express receptors that mark them for quick disposal by macrophages (Savill, 1997).
The most prominent characteristic of the PMN is the multilobulated nucleus. The multilobulated nucleus is important because it allows the PMN to line up its nuclear lobes in a thin line, permitting rapid migration between endothelial cells. Macrophages on the other hand have a large horseshoe shaped nucleus that makes migration between endothelial cells more difficult. Thus, the PMN is the first newly migrated phagocytic cell to arrive at an infection site. Their surface microvilli are also pivotal for their functionality. Within the cytoplasm there are isles of glycogen that make up 20% of the cell on a dry weight basis and numerous bactericidal granules that are used by the cell for bactericidal activity. As in other species, bovine PMN contains azurophilic and specific granules. They also contain a third novel granule that is larger, denser and more numerous than the other two granules. These granules contain lactoferrin, which is also found in secondary granules, but they do not contain constituents common to azurophilic granules. Instead, they contain a group of highly cationic proteins and are the exclusive store of powerful oxygen-independent bactericidal compounds (Gennaro et al., 1983). The most important antibacterial mechanism derived from azurophilic granules is the MPO-H$_2$O$_2$-halide system (Klebanoff, 1970). MPO in the presence of hydrogen peroxide and halide ions kills bacteria. These ROS producing parameters might be altered during physiological and pathological conditions of udder, and should therefore be further investigated.

3. Migration of circulating PMN to the mammary gland

The life cycle of bovine PMN is short. Formed in the bone marrow, PMN require 10 to 14 days to mature (Bainton et al., 1971). After maturation, PMN may be stored for a few additional days. Mature PMN leave the hematopoietic compartment of the bone marrow and enters the vascular sinus by traveling in migration channels through endothelial cells. The PMN circulate in the blood stream briefly (mean half-life of ~9 hours; Carlson and Kaneko, 1975), leave the blood stream by diapedesis and enter tissues where they function as phagocytes for 1 to 2 days. In healthy cows, production and destruction of PMN is tightly regulated, which keeps their number in blood, milk, and tissue almost constant (Paape et al., 1979). Continuous influx of PMN into the mammary gland is an essential element of their role in the first line of immune defense. It is orchestrated by the local accumulation of endogenous and/or exogenous chemotactic factors such as C5a, lipid-derived mediators (e.g., leukotriene B4, Platelet-Activating Factor (PAF) and tissue-derived chemokines (in particular interleukin (IL)-8). The dynamic of PMN diapedesis through blood/milk barrier helps to explain the observed PMN activity fluctuations in milk (Smits et al., 1999).

Although the presence of strong chemotactic factors in non-mastitic milk is debatable, their presence in mastitic milk is indisputable (Manlongat et al., 1998). Most inflammatory chemoattractants are only induced and released during acute infection. However, a restricted number of chemoattractants can be constitutively present in normal plasma at high concentrations, e.g., Regakine-1 (Struyf et al., 2001). During mastitis, inflammatory chemoattractants guide PMN toward infection foci. Potent bovine PMN chemoattractants include C5a, lipopolysaccharides (LPS), IL-1, IL-2 and IL-8 (Gray et al., 1982; Daley et al., 1991; Lee and Zhao, 2000). These chemoattractants bind to specific receptors on the PMN plasma membrane. Extravasation of activated PMN occurs after adhesion of these cells to the endothelial surface. This is accomplished by the expression of specific membrane adhesion molecules. The essential role of the CD (cluster of differentiation) 11/CD18 family of adhesion molecules in PMN-surface adhesion is well-documented (Kishimoto et al., 1989). These molecules bind to endothelial intercellular adhesion molecules (i.e., ICAM-1 and ICAM-2) and endothelial leukocyte adhesion molecules (ELAM-1) on the endothelial surface.
After binding to these molecules, PMN leave the circulation and are ready to function at the infection site. Down-regulated CD11/CD18 in circulating PMN can cause a slower PMN recruitment into the mammary gland. *E. coli* mastitis induces adherence of circulating PMN to the endothelium by up-regulation of CD11b/CD18 (Roets et al., 1999), of which activity is crucial to bovine PMN diapedesis across the blood/milk barrier (Smits et al., 2000). In bovine mastitis, blood PMN number, and effective adhesion, migration, opsonization, phagocytosis and killing are of crucial importance to the outcome of intramammary infection (IMI) and the severity of the disease (Gray et al., 1982; Burvenich et al., 1999a). The impact of fast PMN diapedesis during mastitis on PMN quality and their ROS production capability could cause dissimilarities between milk PMN from inflamed and non-inflamed quarters. The underlying mechanism of this disparity would be pivotal for further investigation.

The source of host and/or pathogen-derived cytokines in milk and their impact on milk PMN function have been a subject of investigation. There is evidence of cytokines secretion by mammary macrophages and epithelial cells during both physiological and pathological conditions of the gland (Politis et al., 1991; 1992; Boudjellab et al., 1998; Okada et al., 1999; Boudjellab et al., 2000). These cytokines influence on PMN function. For example, the interleukin IL-8 is involved with the recruitment of PMN and T lymphocytes into milk (Barber et al., 1999). Pro-inflammatory cytokines, like TNF-α, IL-1β, and lipopolysaccharide (LPS) suppress the gene expression of cytochrome P-450 1A1 (cyplal), by activating the transcription nuclear factor κB (NF-κB) (Ke et al., 2001). PMN also play a crucial role in the recruitment of other leukocytes such as CD4+ T lymphocyte and CD8+ T lymphocyte to the inflammation sites (Solty and Quinn 1999; Montes de Oca et al., 2000). PMN influx to the site of inflammation is important in limiting injury and promoting recovery of severe inflammation (Carey et al., 1997).

The mechanical action of milking enhances PMN diapedesis in milk, which is an important mechanism for PMN surveillance of the mammary gland. Immediately after machine milking, concentrations of PMN in blood from the subcutaneous abdominal vein decrease, while concentrations in mammary lymph and milk increase (Paape and Guidry, 1969; Paape et al., 1985). Thus, the normally sterile mammary gland is provided with millions of PMN for defensive purposes. Besides, newly synthesized milk leads to the removal of migrated PMN and further exudation of PMN into the newly formed milk in the mammary gland alveoli (Schalm and Lasmanis, 1976). However, the ingestion of milk fat globules and casein by PMN results in a loss in phagocytic and bactericidal functions and rapid PMN necrosis (Paape et al., 1975). Conversely, no adverse effect from milk fat globules on C5a chemotactic activity has been reported (Rainard, 2002). In summary, milking removes compromised PMN, which are replaced by new PMN, thus enhancing defense against bacterial infection. Mastitis researchers have long recognized the different types of cells found in bovine milk. These cells include PMN, lymphocytes, eosinophils, macrophages and epithelial cell, all providing somatic cells and mammary defense. Because of the presence of epithelial cells, the term milk somatic cells count (SCC) was coined (Paape et al., 1963). The PMN percentage in normal milk varies from 30 to 100%, depending on the gland status (Paape et al., 1986; Östensson et al., 1988; Burvenich et al., 1994; Suriyasathaporn et al., 2000). PMN also promote tissue injury and disturb mammary function via 1) uncontrolled ROS generation, and 2) granular enzyme release (degranulation) (Nickerson and Heald, 1981; Capuco et al., 1986; Akers and Thompson, 1987; Miller et al., 1993).
4. The LPS-CD14 pathway

The CD14 antigens, which are commonly found on monocytes and macrophages but not on circulating PMN and lymphocytes, were discovered on bovine mammary PMN and macrophages (Paape et al., 1996). The CD14 receptor binds LPS-protein complexes and induces the synthesis and release of TNF-α (Old, 1988). TNF-α up-regulates PMN phagocytosis, adherence, chemotaxis and ROS production. It is now recognized that two forms of CD14 exist, a membrane and a soluble form (Bazil et al., 1986; Haziot et al., 1988). The soluble form results from the shedding of membrane CD14 (mCD14). Soluble CD14 (sCD14) can bind LPS directly and prevent it from binding to mCD14, thus preventing over-secretion of TNF-α that could lead to increased severity of clinical symptoms. The sCD14 has been identified in bovine milk and colostrum as a 46 kDa protein (Filipp et al., 2001; Wang et al., 2002) and may play a role in neutralizing LPS and controlling the clinical symptoms of coliform mastitis. Also, complexes of CD14 and low concentrations (0.2 µg) of LPS induced an increase in milk SCC (Wang et al., 2002). An increase in SCC was not observed after intramammary injection of either CD14 or LPS. It has been reported that LPS-CD14 complexes bind to Toll-like receptors on endothelial and epithelial cells and cause release of IL-8, an important cytokine for recruitment of bovine PMN (Ulevitch and Tobias, 1999). Transgenic mice carrying the gene for CD14 in their mammary cells are currently being developed by scientists in the Gene Evaluation and Mapping Laboratory at the USDA in Beltsville. Experimental infections with E. coli will be conducted to see if the secreted rbosCD14 will complex with the LPS produced by E. coli and result in recruitment of PMN in mammary gland and elimination of the bacteria.

5. LPS detoxification in the mammary gland

Endotoxins or LPS are released during bacterial growth and lysis of Gram-negative bacteria and have been recognized as important mediators for the treatment and outcome of coliform mastitis (Lohuis et al., 1988a; b; Pyörrälä and Syväjärvi, 1987). The role of the absorption of free LPS into the circulation is controversial (Dosogne et al., 2002). In contrast, it is accepted that the amount of released LPS into the mammary gland, its subsequent detoxification and TNF-α production significantly contribute to the outcome of coliform mastitis (Blum et al., 2000; Hoeben et al., 2000b). Severity of E. coli mastitis seems to be related to the enhanced release of secondary induced inflammatory mediators such as TNF-α (Blum et al., 2000), as a result of impaired LPS detoxification mechanisms in milk. It has been suggested that local CD14 expression modulates the toxic effects of LPS in the mammary gland (Burvenich et al., 1996; Paape et al., 1996).

Another detoxification system is acyloxyacyl hydrolase (AOAH), an enzyme produced by PMN that hydrolyses LPS. AOAH is also present in bovine PMN granules (McDermott et al., 1991) and hydrolyses two acyl chains of the lipid A of endotoxin, resulting in a decreased toxicity whereas the immunogenic properties of LPS are largely maintained (Munford and Hall, 1986). Little has been investigated about milk PMN AOAH activity either during physiological or mastitis conditions. Immediately after calving, there is a decreased blood
PMN AOAH activity (Dosogne et al., 1998) that coincides with the decreased PMN ROS production and number in circulation (Moreira da Silva et al., 1998). This coincidence could be considered as a risk factor for coliform mastitis during early lactation. Indeed, intravenous LPS administration to rabbits resulted in a rapid (within 90 min) increase of plasma AOAH activity (Erwin and Munford, 1991). The finding that PMN AOAH activity is increased upon LPS stimulation may indicate the existence of a PMN-dependent self-regulatory protection mechanism against endotoxemia. Study on “milk” PMN AOAH activity would be interesting for assessment of severity of coliform mastitis.

Besides AOAH, bovine PMN granules also contain different LPS binding cationic proteins such as lactoferrin, and a huge variety of cationic antimicrobial proteins (Levy et al., 1995). These proteins do not degrade the LPS molecule, but binding to LPS results in a decreased LPS bioavailability and hence may attenuate its toxicity during Gram-negative bacterial infections. In a recent study, oral lactoferrin administration attenuated spontaneous TNF-α production by peripheral blood cells in human (Zimecki et al., 1999). Study on the relation between PMN AOAH activity, ROS production and viability and severity of coliform mastitis in dairy cows would be very interesting for the physiopathology of coliform mastitis.

6. Bactericidal mechanism of PMN

Following adherence of opsonized bacteria to surface receptors on the PMN, phagocytosis, respiratory burst and degranulation are triggered. The process of opsonization, though not essential for phagocytosis, certainly promotes the uptake of bacteria by PMN. The phagocytosis process is energy-dependent and requires the presence of a functional cytoskeleton. The cytoskeleton machinery, when sequentially activated following receptor stimulation, is thought to envelope the microorganism in a “zipper mechanism” (Griffin et al., 1975). Immunological recognition is mainly accomplished by specific antibodies (IgG2 and IgM) which recognize the bacterium through Fab-regions and bind to PMN via Fc-receptors on the PMN plasma membrane (Paape et al., 1991). There is a synergy between the Fc and C3b receptors activity and PMN ROS production (Newman and Johnston, 1979).

Diapedesis will also affect binding of immunoglobulins to the PMN surface. Targowski and Niemialtowski, (1986) observed an increased expression of Fc receptors and phagocytosis after in vitro migration of bovine PMN through membranes. This was confirmed in further studies by Berning et al. (1993) and Worku et al. (1994). After in vivo migration of PMN into mammary quarters of nulliparous heifers, binding of IgG1 and IgG2 increased while binding of IgM decreased. Binding of IgA remained unchanged. The greatest change occurred with the binding of IgM. Seventy-six percent of the blood PMN bound IgM, whereas only 2% of the mammary PMN bound IgM. Interestingly, phagocytic activity of PMN increased after in vitro chemotaxis but not after in vivo chemotaxis. Activation of complement also promotes phagocytosis and killing. The C3b and C3bi, generated on the surface of bacteria following antibody union, are recognized respectively by CR1 and CR3 receptors located on the PMN cell membrane. The type of bacteria also affect bovine PMN bactericidal capacity. For example, slime-producing \textit{Staphylococcus aureus} hampers the killing capacity of PMN (Barrio et al., 2000). The specific interactions between extracellular matrix proteins of \textit{Staphylococcus aureus} and ICAM-1 inhibits further PMN recruitment, boosting anti-inflammatory reactions (Chavakis et al., 2002). This might be counterproductive for the killing activity of PMN. During migration of PMN into milk in response to infection increased binding of C3b was observed (DiCarlo et al., 1996). Thus, PMN are fully armed to confront invading bacteria, resulting in a more rapid ingestion and elimination of the pathogens. Once complement and immunoglobulins bind to receptors on the PMN surface, PMN become
activated and generates ROS, such as superoxide anions \( (\text{O}_2^-) \), hydrogen peroxide \( (\text{H}_2\text{O}_2) \) and halogen reactive species (Leino and Paape, 1993). This process associated with the respiratory burst is called the “oxygen-dependent” or “oxidative” killing, and that associated with neutrophil granules is also called “oxygen-dependent”, but “non-oxidative” killing. Killing classified on the basis of these criteria has been explicitly reviewed (e.g., Root and Cohen, 1981; Babior, 1984; Spitznagel and Shafer, 1985, Bertram, 1985 and Babior, 1994).

Intracellular killing of phagocytosed microorganisms is accomplished following adherence to the PMN surface, usually, but not necessarily, via specific receptors (Horwitz et al., 1982). Three different mechanisms are involved for intracellular bacterial destruction: 1) an oxygen-dependent mechanism (production of reactive oxygen compounds), 2) a nitrogen dependent mechanism (nitrogen oxide derived from L-arginine) and 3) an oxygen-and nitrogen-independent microbicidal mechanism e.g., lysozyme, lactoferrin, proteases, pH changes. In this study our prime focus is the oxygen-dependent microbicidal mechanism.

Baldridge and Gerard (1933) first reported that an increase in oxygen consumption by neutrophils takes place when phagocytosis is triggered. ROS generated by reduction-oxidation (redox) reactions, have been recognized as one of the major contributors to the killing of pathogens. This phenomenon is accompanied by an increase in oxygen consumption and the hexose monophosphate shunt (HMPS) activity by PMN and has been termed the “respiratory” burst. The oxygen molecule is central for PMN respiratory burst activity (Allen et al., 1972; Babior, 1984; 1994); its importance in microbicidal activity of PMN was highlighted by the inefficient PMN bactericidal activity in anaerobic conditions (Mandell, 1974). One example: for each molecule of \( \text{O}_2 \) consumed 4 \( \text{O}_2^- \) ions are generated; roughly 0.5 fmol of \( \text{O}_2 \) is consumed for each bacterium engulfed, resulting in an intravacuolar \( \text{O}_2^- \) release of about 4 mol.l\(^{-1} \) (Reeves et al., 2002). Though still remains inconclusive, the application of ozone gas (\( \text{O}_3 \)) would be further examined in dairy cows for treatment of clinical mastitis; because it boosts milk PMN ROS production capacity (Ogata and Nagahata, 2000).

The first step in the cascade of respiratory burst is the formation of \( \text{O}_2^- \), requiring NADPH-oxidase, of which substrate (NADPH) is generated by HMPS to act as an electron donor (Rossi and Zatti, 1964; Babior, 1984; Rossi, 1986).

\[ 2 \text{O}_2 + \text{NADPH} \rightarrow 2\text{O}_2^- + \text{NADP}^+ + \text{H}^+ \]

Different stimuli (e.g., complement components, immunoglobulin, formyl-methionyl-leucyl-phenylalanine (fMLP), phorbol myristate acetate (PMA) and bacterial peptides) act via different specific receptors and thus have various signal transduction mechanisms to activate the NADPH-oxidase. Extensive research into activation by PMA has followed the identification of protein kinase-c (PK-C) as its cytosolic receptor (Nishizuka, 1984). PMA is a strong NADPH-oxidase and PK-C agonist (Tauber, 1987, Karlsson et al., 2000). Particulate stimuli may also act indirectly via PK-C (Cooke and Hallett, 1985) or via other intermediates such as arachidonic acid and its metabolites and phospholipase-A2 (Tauber, 1987). The next step is the formation of \( \text{H}_2\text{O}_2 \) by dismutation of \( \text{O}_2^- \), which is mediated by superoxide dismutase (SOD):

\[ 2 \text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \]

The \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \), generated by NADPH-oxidase and SOD, are the precursors of variety of subsequent powerful ROS. Included among these ROS are a variety of oxidized halogens, including hypohalite ions or HOX (Thomas and Fishman, 1986; Weiss et al., 1986) and a variety of chloramines (Thomas et al., 1982) used by PMN as microbicidal agents. These are generated by the \( \text{H}_2\text{O}_2 \)-mediated oxidation of halide ions under catalysis by MPO or eosinophil peroxidase (EPO) and the subsequent oxidation of amines:
Though not as crucial as PMN in mastitis, the activity of compound I of EPO to react with H$_2$O$_2$ is similar to that of MPO but with substrates like Cl$^-$, however, it is far higher, yielding more HOX (Arnhold et al., 2001).

Another group of ROS that are produced from O$_2^\cdot$ are the hydroxyl radicals (OH$^\cdot$) generated in a transition metals (Fe or Cu) catalyzed reaction between O$_2^\cdot$ and a hydroxyperoxide (a well-known Haber-Weiss reaction, if R = H):

\[
O_2^\cdot + ROOH \rightarrow RO + OH^\cdot + H_2O_2 \quad (R = H, -C, C(=O))
\]

or in a reaction between previously generated oxidizing radical and another compound:

\[
OH^\cdot + RH \rightarrow R^\cdot + H_2O
\]

Eventually, singlet oxygen (1O$_2$) has been found to be produced by PMN and eosinophils (see e.g., Root and Cohen, 1981; Allen et al., 2000), possibly through a reaction between hypohalyte and H$_2$O$_2$:

\[
HOX + H_2O_2 \rightarrow 1O_2 + X^- + H_2O
\]

It is evident that the production of large quantities of ROS with a cascade of reactions will provide an environment that is destructive for any microorganisms exposed to it, but it is also harmful to the nearby tissues. That is to say that ROS represent a “double-aged sword”. Alternatively, ROS also enhance natural killer cell and T cell activity (Suthanthiran et al., 1984; Cemerski et al., 2002; Reth, 2002), indicating that PMN ROS may not only damage cells and tissues but may also accelerate recovery of inflammation. The above reactions are tightly regulated so that the PMN releases its ROS under appropriate circumstances, depending on the physiological and pathological conditions of animal. What is not yet clear is whether there is a transient PMN ROS production change during physiopathological conditions, and if so, whether this change is or is not beneficial for animals.

PMN are capable of producing a range of ROS following activation of the membrane bound NADPH-oxidase. It is also generally agreed that ROS boost oxygen-independent microbicidal activity (Reeves et al., 2002). Root and Cohen (1981) have suggested several possible direct sites of action for ROS, related to their microbicidal activity; these include: 1) unsaturated carbon bounds that may lead to toxic lipid peroxidation, 2) sulphydryl groups that lead to the destruction of sulphydryl containing enzymes, 3) amino group and possible peptide bound breakage and 4) nucleic acids. In vitro studies with O$_2^\cdot$ generating systems such as xanthine oxidase (Rosen and Klebanoff, 1976) suggests that O$_2^\cdot$ are far more toxic to bacteria if they operate in MPO H$_2$O$_2$-halide system, which leads to the production of powerful chlorinated oxidising agents such as ClO$^-$ which have a bactericidal effect by halogenating bacterial proteins.

Several techniques of PMN ROS quantification are frequently applied. For example, the cytochrome c reduction test, flow cytometry method (Salgar et al., 1991), the scopoletine test (Root et al., 1975) and CL assay (Allen et al., 1972; Weber et al., 1983; Piccinini et al., 1999; Hoeben et al., 2000a; Mehrzad et al., 2000a). CL simplifies PMN ROS production measurement, and is a relatively recent technique. Application of CL technique to study PMN function helps to gain more insight into first line of immune defense mechanisms and the pathophysiology of infectious diseases related to early post-calving period. For CL quantification, we need viable PMN, a PMN activator (e.g. PMA, fMLP, particles, etc.) and a CL substrate (e.g., luminol, lucigenin etc). Luminol-dependent CL has been described as an appropriate probe for assessment of blood and milk PMN ROS production (Briheim et al., 1984).
The PMN metabolic pathways responsible for O₂-dependent bactericidal activity and CL are depicted schematically in figure 1.

Figure 1. Diagram depicting the major enzymatic systems responsible for microbicidal metabolism and oxygenation activities and the relationship of these activities to photon emission. In the scheme the activities of the cytoplasmic milieu are separated from those of the phagosome-phagolysosome-extracellular milieu. The superscripted number that precedes each molecular symbol (e.g., 1 for singlet, 2 for doublet, and 3 for triplet multiplicity) depicts the equation: $|2s| + 1 = \text{multiplicity (n)}$. The diagram adapted from (Allen et al., 2000).
7. Periparturient PMN dysfunction

Reduced functional competence of PMN has been associated with decreased immunocompetence, resulting in an increased susceptibility to infection and suppression of host defense mechanisms. A dramatic reduced random migration, iodination and chemiluminescence of blood PMN were observed during the first week after parturition (Kehrli et al., 1989; Heyneman et al., 1990; Kremer et al., 1993; Vandeputte-Van Messom et al., 1993; Shuster et al., 1996; Hoeben et al., 2000a; Dosogne et al., 2001). It was recently discovered that the adhesion molecule L-selectin is shed from the surface of PMN at parturition (Lee and Kehrli, 1998). Surface expression of L-selection remains low for several days following parturition and could contribute to the reported defect in bovine PMN chemotaxis during the period immediately following calving (Berning et al., 1993). Regulation of bovine PMN adhesion molecules during mammary gland infection and possible use of immunomodulators has recently been reviewed (Diez-Frail et al., 2001). Cumulative deficiencies in opsonin levels (IgG1 and conglutinin) were observed in periparturient cows, which closely coincided with impaired PMN oxidation-reduction reactions capacity (Detilleux et al., 1994). The proportion of all cases of clinical coliform mastitis that develop during the first 8 weeks of lactation has been reported to be around 50% (Burvenich et al., 1994; Pyörälä and Pyörälä, 1998). At the moment the underlying mechanisms involved in periparturient immunosuppression remain unknown. However, metabolites (e.g., β-hydroxybutyrate) (Suriyasathaporn et al., 1999) and hormones (e.g., growth hormone, cortisol, pregnancy associated glycoprotein) (Gray et al., 1982; Kremer et al., 1993; Persson et al., 1993; Burvenich et al., 1996; Suriyasathaporn et al., 2000) have been reported as attributable factors.

8. Hormones and metabolites versus PMN ROS production and viability

There are many studies demonstrating the link between periparturient immunosuppression and hormonal-and-metabolic changes, directly/indirectly contributing to PMN ROS production capacity (Gray et al., 1982; Kremer et al., 1993; Persson et al., 1993; Burvenich et al., 1996; Suriyasathaporn et al., 1999). For example, glucocorticoids, ketone bodies and pregnancy associated glycoproteins play a causative role in impaired PMN ROS production capacity (Moreira da Silva et al., 1998; Hoeben et al., 2000a). These hormones and metabolites also inhibit the proliferation of bone marrow cells in vitro (Hoeben et al., 1999). Our understanding of the precise ways in which the complex cascade of ROS production occurs in blood or milk PMN during physiological and mastitis conditions is still in its early stage. This is especially true for the in vivo effect of PMN ROS and viability by hormones and metabolites. Recombinant bovine somatotropin (bST) has been shown to boost cows’ milk production (MP) and compositional performance following experimentally induced E. coli and Streptococcus uberis mastitis (Hoeben et al., 1999). Recombinant bST also prevents severe local and general clinical symptoms in cows suffering from E. coli mastitis. Prolactin, bST, and insulin-like growth factor-I (IGF-I) are thought to be involved in several immune functions (Elvinger et al., 1991; Hoogehe et al., 1993; Adriaens et al., 1995; Kooijman et al., 1996). The function of bST on PMN can either be directly or indirectly mediated through IGF-I. Plasma and milk concentrations of
IGF-I increase after bST administration (Cohick et al., 1989; Mielke et al., 1990; Vicini et al., 1991; Burton et al., 1992; Zhao et al., 1992). Their concentration differs throughout lactation in milk (Campbell et al., 1991; Baumrucker et al., 1993). Massart-Leën et al. (1990) reported an increased number of circulating leukocytes, band neutrophils, and an enhanced PMN ROS production in cows treated with bST after calving. PMN ROS generation, chemotaxis, random migration, phagocytosis towards IgG-opsonised micro-organisms is boosted by IGF-I and bST (Edwards et al., 1988; Kelley, 1989; Fu et al., 1991; Edwards et al., 1992; Jin et al., 1993; Wiedermann et al., 1993; Fu et al., 1994; Bjerknes and Aarskog, 1995; Warwick Davies et al., 1995). The expression of complement receptors can be upregulated by bST and IGF-I. Increased chemotaxis and random migration of PMN (Wiedermann et al., 1993), increased numbers of circulating neutrophils (Clark et al., 1993), and increased proliferation of granulocyte and monocyte precursors (Merchav et al., 1988; Scheven and Hamilton, 1991; Merchav et al., 1993) have also been observed following bST and IGF-I elevations in vivo. Elvinger et al. (1991) reported little or no effect on phagocytosis and killing of E. coli by circulating PMN or on cytochrome c reduction in vitro as well as in vivo. However, Heyneman and Burvenich (1989) observed an increased PMN ROS production capacity after in vivo administration of bST in healthy cows. Similar results were observed in vitro by us (Mehrzad et al., 2002). There may be economic and public health debate over the use of hormones (such as, steroids, bST, IGF-1) and we might not suggest its application in food animals. On the other, concentrations of these hormones fluctuate significantly during physiological and pathological conditions of animal, suggesting that basic study on this topic might narrow bridge between hormone and first line immune defense mechanism. One example: concentration of β-lactoglobulin, as a booster for milk PMN ROS production (Merzad et al., 2000a) and general immunostimulator (Wong et al., 1998), is lowest during early lactation (Caffin et al., 1984). This helps us to clearer interpret our finding on potential immunomodulation and/or immunosupression conditions occurring throughout lactation cycle. Whether there is a positive correlation between milk PMN ROS production capacity and their viability during (patho)physiological condition of the mammary gland would be important assumption.

9. Blood and milk PMN ROS production versus lactation, parity and mastitis

Blood and milk PMN have the potential to produce substantial amounts of ROS to kill engulfed bacteria (Weber et al., 1983; Dulin et al., 1988; Hallén Sandgren et al., 1991; Piccinini et al., 1999; Hoeben et al., 2000a). Whether ROS production of resting (non-stimulated) and stimulated milk PMN is correlated to their counterparts in blood is still a question. As CL widely used technique for PMN ROS production assessment (Allen et al., 1972; Weber et al., 1983; Piccinini et al., 1999; Hoeben et al., 2000a), it results from reactions of ROS with luminescence agents, requiring both cytoplasmic membrane NADPH-oxidase and MPO in the azurophilic granules of the PMN (Webb et al., 1974; Babior, 1984). During the last years little comparison has been made between CL of PMN in blood and milk. To interpret and assess the responsiveness of PMN to stimulating agents such as PMA, it is necessary to distinguish between stimulated and non-stimulated PMN. This offers information about the activity of protein kinase C and NADPH-oxidase, as PMA is a protein kinase C and NADPH-oxidase agonist (Karlsson et al., 2000). For example, whether CL of resting milk
PMN differs from that of blood PMN; ingestion of milk fat globules and casein micelles affects milk PMN quality (Russell and Reiter, 1975; Paape and Guidry 1977; Dulin et al., 1988) and subsequent degranulation (Russell et al., 1976; Paape and Wergin, 1977a). No such a problem in blood is existed. Smits et al. (1999) have shown that diapedesis of PMN across mammary epithelium in vitro reduces ROS production of PMN, suggesting milk PMN function might differ from their blood counterparts (Paape and Guidry 1977; Smits et al., 2000). Potential physiological influencing factors such as stage and number of lactation might be involved in this cell impairment. One example would be β-lactoglobulin (Mehrzad et al., 2000b), of which concentration in milk is minimal during early lactation (Caffin et al., 1984). The PMN function impairment generally coincides with cow’s susceptibility for environmental mastitis (Burvenich et al., 1994; Shuster et al., 1996). Increased severity of dairy cows to E. coli mastitis has also been associated with increased parity (van Werven et al., 1997). Strong inhibition of fMLP-stimulated ROS production was observed following exposure of PMN to a high IgG concentration, indicating that the IgG interferes the activation of phosphatidylinositol (PI) 43-kinase but not of PK-C (Yue et al., 2001). This also indicates that the IgG complexes can penetrate cell surface and enter into PMN cytosol by endocytosis.

The luminol-dependent CL system requires H2O2 (Lind et al., 1983; Faulkner et al., 1993), preferentially quantifying intra-and extracellular ROS (Briheim et al., 1984). This offers information about PMN intracellular H2O2 production capacity. Low bactericidal activity of PMN in early lactation dairy cows (Dosogne et al., 2001) might be due, in part, to lower intracellular ROS production. Substantial evidence exists on the blood PMN ROS production capacity fluctuation during mastitis (Heyneman et al., 1990; Vandeputte-Van Messom et al., 1993; Hoeben et al., 2000b). However, little investigation has been conducted on milk PMN ROS production capacity during LPS and/or E. coli mastitis. Therefore, fundamental study on this topic is needed and the current study provides some insight into milk PMN ROS production versus mastitis.

10. PMN viability and apoptosis in milk

Till now, little attention has been paid to milk cell viability and apoptosis. It might be acceptable that the survival of PMN in blood stream is constant. However, diapedesed PMN in milk, have a different life span. More importantly, an effect of stage and number of lactation on viability of “milk” PMN could be expected and would provide fundamental insight into the physiological influencing factors on the quality of milk PMN. Furthermore, the mechanism of these effects would be interesting to investigate. According to the studies on human and bovine PMN (Mayer et al., 1989; Jankowski et al., 2002), contribution of neutrophil NADPH-oxidase activity to viability is pivotal. This enzyme activity contributes to phagosomal and cytosolic pH homeostasis. There might be a link between “milk” PMN ROS production and viability; O2\(^-\) together with PMN granule contents exerts cytosolic pH buffering effects. These buffering effects are critical for PMN viability (Takanaka et al., 1988; Mayer et al., 1989; Jankowski et al., 2002; Reeves et al., 2002), suggesting a central role of NADPH-oxidase activity on PMN viability. NADPH-oxidase activity is regulated by MPO (Edwards et al., 1986). There can be other contributing physiological factors which remain to be investigated. Preliminary evidence supports
the hypothesis of rapid and profound cytosolic acidification, which could result in faster PMN necrosis. During experimentally induced mastitis rapid migration of circulating PMN into the mammary gland occurs (Ferrante, 1992; Lee et al., 1993), resulting in delayed PMN apoptosis and increased viability (Watson et al., 1997). The speed of PMN diapedesis in milk during mastitis could affect PMN survival in inflamed quarters. The inflammation of the mammary gland provokes local and systemic effects (Pyörälä and Syväjärvi, 1987). The systemic effect of mastitis could affect the viability of non-infected milk PMN. This results mainly from recruitment of younger neutrophils in non-infected quarters. The contribution of blood-milk barrier (Van Oostveldt et al., 2002a) and mammary gland injury (Sladek et al., 2001; Van Oostveldt et al., 2002b) in the modulation of PMN apoptosis has been recently reported. The lactation-and-parity-related milk PMN viability alterations can be identified as a very complex and interesting issue. The milk PMN viability impairment might be a potential culmination of immunosuppression of the mammary gland. The question of whether higher milk PMN viability contributes to a better protection of the mammary gland against invading pathogens is another considerable subject of the udder’s defense. The study of PMN viability and apoptosis could help bovine immunologists to get a better insight into the pathophysiology of mastitis. The latest research in human (Vissers et al., 2001) uncovers the ability of intracellular vitamin C to protect PMN from apoptosis and necrosis against HOCL and toxic chloramines. This protection resulted from altering caspase activity (Fadeel et al., 1998; Akgul et al., 2001), as caspase activation is a known apoptosis-processing signaling pathway. In healthy cow the most interactive approaches of PMN viability would be interaction between ROS generation and caspase activity of milk PMN.

11. Genetic factors affecting PMN function and outcome of mastitis

Heritabilities ($h^2$) in mice ($h^2 = 0.26$) and Yorkshire pigs ($h^2 = 0.20$) (Biozzi et al., 1998) from selective breeding for high and low immune responses, based on multiple antibody and cell-mediated immune responses traits, demonstrated that inflammation is controlled by genetic factors. The inflammatory response is associated with protective mechanisms, among which is the production of heat shock protein (Hsp) involved in PMN apoptosis and regulation of cell proliferation. In cattle, genes coding for several Hsp70s have already been mapped (Wilkie et al., 1999). The similarities found between PMN recognition mechanisms for pathogens and apoptotic PMN suggest that similar genes are involved in both processes and provide new direction in research for genetic resistance to disease (http://bos.cvm.tamu.edu/bovgbase.html; http://locus.jouy.inra.fr/cgi-bin/bovmap/intro.pl).

In dairy cows, the concentration of circulating PMN during early lactation is highly heritable ($h^2 = 0.87$; Riollet et al., 2000). The importance of adhesive interactions between bovine PMN and the endothelium is revealed by the impaired random migration and phagocytosis of PMN of cattle homozygous for the recessive allele that is responsible for leukocyte adhesion deficiency (BLAD), the gene coding for the β-subunit of the CD18 adhesion molecule (Franc et al., 1999). Kelm et al. (1997) found an association between the presence of this allele in heterozygous cows and a decrease in their clinical mastitis genotypic value. However, the frequency of infected and clinical mastitic quarters was not different between heterozygous carriers and homozygous normals (Wagter et al., 2000). The PMN-endothelium interaction results
in a number of intracellular signalling events that have homologies in *Drosophila* and plants. In mice and humans, mutations in Toll-like transmembrane receptor genes have been associated with susceptibility to Gram-negative infections (Wanner et al., 1999). Inhibitors of intracellular tyrosine kinases reduce internalisation of *S. uberis* by mammary epithelial cells (Qureshi et al., 1999). The transcription nuclear factor, NF-kB, the end product of this intracellular pathway, regulates the expression of a wide variety of genes involved in inflammation and immunity. It is believed that Toll receptors and the associated signalling pathways of NF-kB may represent the most ancient host defense system found in mammals, insects and plants. These discoveries may facilitate the genetic dissection of the innate inflammatory reaction to infection by allowing meaningful cross-species comparison and analysis of newly discovered genes.

Genotypic values for clinical mastitis are lower in Holsteins bearing the IgG2a immunoglobulin isotype than in cows bearing the IgG2b allotype (Kelm et al., 1997). In post-parturient Holstein cows (h² = 0.41) and in non-parturient Norwegian Red cattle (h² = 0.75), haemolytic complement activity is heritable (Riollet et al., 2000). Genes coding for complement components 1, 4, 5 and 9 have been mapped to different bovine chromosomes (Wilkie and Mallard, 1999). The PMN ingestion process may not be essential for the outcome of IMI, because percentage of *E. coli* ingested by blood PMN before experimental *E. coli* infection is not statistically associated with the decrease in milk production in the non-infected gland (Dosogne et al., 1997). Also, percentage of *S. aureus* ingested by PMN is not heritable (Burvenich et al., 1999b) during the periparturient period. On the other hand, heritability for cytochrome C reduction (h² = 0.22 around parturition) and for stimulated CL activity (h² = 0.30 around parturition) are high (Riollet et al., 2000). Negative genetic correlations exist between SCC genotypic values and blood PMN stimulated CL (r_g = -0.26) and between IMI genotypic values and blood PMN cytochrome C reduction (r_g = -0.33 before parturition) (Kelm et al., 1997). Strains of mice lacking the PMN elastase (oxygen-independent antimicrobial agent) gene are more susceptible than their normal littermates to sepsis and death following intraperitoneal infection with *E. coli*, but not with *S. aureus* (Belaouaj, et al., 1998). Bovine lysozyme and lactoferrin genes have been identified (Wilkie and Mallard, 1999), and bovine milk concentration of lactoferrin is moderately heritable (h² = 0.4) (Mallard et al., 1998). Genetic selection for cows resistant to mastitis could become an important alternative for prophylactic measures of mastitis in the future.

In summary, based on what is known so far, it is conceivable that the defense of the mammary gland against mastitis-causing pathogens could be mediated by PMN ROS production and milk PMN viability. Both of these parameters might be affected by physiological (e.g., stage of lactation and lactation number) and pathological (endotoxin and *E. coli* mastitis) conditions of dairy cows. Milk PMN ROS production load and viability at the start of bacterial invasion can be considered as two major attributable factors for phagocytosing and intracellular killing of invading bacteria, which influence on the outcome of mastitis. These PMN functionality fluctuations and mastitis outcome are multifactorial and can be affected by speed of diapedesis and complex chemical environment of milk compartment. Substantial fundamental studies are required on immunophysiological status of dairy cows. This thesis was performed to gain more insight into the complex events of first line immune defense mechanisms and the pathophysiology of mastitis related to early post-calving period.
References


Objectives
Objectives

The general objective of this thesis is to evaluate the reactive oxygen species (ROS) production and viability of polymorphonuclear leukocytes (PMN) from high yielding dairy cows during several physiological and mastitis conditions.

Blood and milk PMN will be studied during physiological conditions such as stages of lactation (chapter 4), around parturition and in different parities (chapter 5) and during pathological conditions i.e. endotoxin (chapter 6), *E. coli* (chapter 7) mastitis and mastitis severity (chapters 6 and 8). Figure 1 gives a schematic overview of the objectives of this thesis.

Figure 1. Schematic overview of the objectives of the thesis. The parameters studied throughout the thesis are: blood and milk PMN maturity, diapedesis, viability, ROS production and bactericidal capacity under both physiological (parturition, stage of lactation and parity) and pathological (endotoxin and *E. coli* mastitis) conditions. The relation of these parameters to the severity of mastitis will also be investigated.
Hypothesis

Formed in bone marrow, polymorphonuclear leukocytes (PMN) are one of the most important first line of immune defense mechanisms in the dairy cow. PMN are the only leukocytes in milk capable of producing a large amount of reactive oxygen species (ROS) to kill engulfed microorganisms. Impaired PMN function is a physiological phenomenon observed in healthy-periparturient cows.

We propose that the PMN ROS production impairment is not only linked to parturition but also to parity, and potentially contribute to a higher risk for mastitis in dairy cows during early lactation.

To test this hypothesis, the chemiluminescence kinetics of blood and milk PMN were studied during different stages of lactation, different parities and in different-mastitis models. In addition, it was evaluated whether the changes in ROS production coincide with changes in blood and milk PMN viability and/or diapedesis.
Part 1

Development and evaluation of the methods
Chapter 1

Isolation, differentiation and quantification of blood and milk neutrophils

This chapter is based on:


Abstract

Polymorphonuclear leukocytes (PMN) are crucial in both preventing and protecting dairy cows from infection such as mastitis (Burvenich et al., 1994; Kehrli et al. 2001). Whereas many differential leukocyte count methods for blood leukocytes are available, little have been developed for milk leukocytes. To unequivocally evaluate PMN functional assay, an appropriate isolation, differentiation and quantification of leukocytes in original or purified samples are essential. This is more special for non-mastitis milk leukocytes; not merely because a variety of cells e.g., PMN, macrophages, lymphocytes and epithelial cells, are existed but because their shapes, size and population could differ, compared to the blood. Even for blood leukocytes, their shapes and population changed significantly during mastitis. All of these changes could interfere with the assessment of PMN function. To overcome any problem and to simplify PMN functional assay, we studied blood and milk cell isolation, differentiation and quantification.
1. Introduction

Polymorphonuclear leukocytes (PMN) is crucial compartment of first line of immune defense in dairy cows and predominant blood leukocytes during mastitis in mammary gland (Burvenich et al., 1994). The real issues in milk and beef industries are many. Among them is mastitis, which is current’s increasingly focus of many bovine immunologists. Although the important role of blood PMN in the pathogenesis of mastitis is well described, the contribution of resident milk cells to the intramammary (i.mam.) defense mechanism is not well characterized. In healthy as well as mastitis cows, the number, differential count and function of leukocytes within the mammary gland could contribute to the defense against invading pathogens. Both naturally occurring (Vandeputte-Van Messom et al., 1993; Shuster et al., 1996) and artificially induced (Nickerson et al., 1990) increased SCC have been shown to exert a protective effect against the severity of the inflammatory response to i.mam. infections. So far, study on the qualitative role of milk leukocytes in healthy and mastitis cows is rare. The milk leukocytes differentiation also appears difficult. In addition, little attention has been paid to the standardization of particularly sample preparation procedures. Milk sample processing varies from the use of centrifuged whole milk samples (Redelman et al., 1988) to dilution with a hypotonic buffer (Hageltorn and Saad, 1986; Östensson et al., 1988). Without microscopic confirmation, flow cytometric identification of bovine milk cells based on forward and side scatter is inconclusive because phagocytosis of milk components may alter both size and intracellular granularity. Cellular debris may also interfere with the scatter pattern of normal cells. Therefore, this study was objected to develop an isolation, differentiation and enumeration of leukocytes in blood and milk to better assess PMN function in blood and milk.

2. Materials and methods

Animals
Thirty healthy Holstein-Friesian cows (first to third lactation; day 35±4 to 170±20 of lactation) from the Ghent University dairy farm (Biocentrum Agri-Vet Melle, Belgium) were investigated for blood and milk sampling, some of which were experimentally induced mastitis. Cisternal quarter milk samples (500 ml) were aseptically collected, after cleaning and disinfection of the teats, using a sterile teat cannula according to Vangroenweghe et al. (2000) and stored on ice for cell isolation. Simultaneously after milk sampling, blood samples (80 ml) were aseptically collected from the external jugular vein into evacuated tubes (Laboratoire EGA, Nogent-le Roi, France) containing 125 I.U. heparin for further process.

Blood and milk PMN isolation
Isolation of PMN from peripheral blood was performed applying hypotonic lysis of erythrocytes according to Carlson and Kaneko (1973). Milk PMN isolation was performed using a high-capacity centrifuge (RC-3BP, Sorvall, Newtown, CT, USA) after 60 % v/v dilution with cold phosphate buffered saline (PBS; 0.01 M phosphate buffer (KH2PO4-Na2HPO4)-0.15 M NaCl, pH = 7.2). After carefully removing of fat with centrifugation (600 × g, 15 min, 4°C), the pellet was washed twice at 300 × g for 10 min and 200 × g for 15 min at 4°C, in cold PBS, the cells were finally resuspended in DPBS supplemented with gelatin 0.5 mg / ml for differentiation and enumeration.
To further purify milk isolated cells (especially, PMN and macrophages), a Percoll isolation procedure was used, applying 3 ml of 2 different Percoll (Pharmacia Biotech AB, Uppsala, Sweden) solutions with densities of 1.092 and 1.071 g/l; these were layered in a 10 ml test tube (see figure 2). This gradient was carefully overlaid with 3 ml of a milk cell suspension and centrifuged at 400 × g for 25 min at 4°C. Upper, middle and bottom layers were obtained. Fractions were collected individually and washed (200 × g for 10 min at 4°C), counted and differentiated. Heterologous plasma was added to the isolated cells in a 1:1 ratio to prevent lysis during preparation of the smears.

**Blood and milk PMN differentiation**

Ten µl of homogenized whole blood was added onto the microscope slides, and very thin smears were prepared accordingly. The smears allowed air-dried for 1 h. Subsequently, the smears used for staining procedure. Briefly, staining was performed on the smears using rapid method of eosin-Giemsa staining procedure: five seconds in the methanol for fixation, 5 seconds in eosin and 7 seconds in Giemsa (Hemacolor® Merk Diagnostics, Darmstadt, Germany) followed by washing first with tape water and finally with double distilled water. After air drying, the smears were used for differentiation using light microscopy (Axioscop 2 MOT; with digital camera, Axiocom; connected to the computer program Axiovision 3.0 Manual; Ziezz, Germany) with the magnification of ×1000, for taking image.

A described procedure for making microscope slides for isolated blood and milk cells was used (Dulin et al., 1982). Slides were prepared in a cytocentrifuge (Shandon, UK), using mixed of 30 µl cell suspension and 30 µl plasma with a concentration between 1×10⁶ and 5x10⁶ cells/ml. The suspensions were centrifuged during 20 seconds at 350 x g. Differential cell counts on blood and milk isolates were performed on eosin-Giemsa-stained smears (Hemacolor® Merk Diagnostics, Darmstadt, Germany) using light microscopy. Identification of the cells in isolates and whole blood was based on morphological characteristics as described by Hayhoe and Flemans, (1969). Cells differentiation in milk was also based on morphological characteristics as described by McDonald and Anderson (1981). PMN (mature and immature), monocytes/macrophages (M), lymphocytes (L) and epithelial cells (negligible in milk “isolates”) were identified on 200 cells per slide.

To compare the surface morphology of PMN, isolated blood and milk PMN were exclusively prepared for scanning electron micrograph examination. This assay was performed using scanning electron microscopy (JEOL, JSM-500 LV).

**Cell enumeration in blood and milk**

The total number of blood leukocytes and isolated cells from blood and milk were determined using an electronic particle counter (Coulter counter Z2, Coulter Electronics Ltd., Luton., UK). To do this, 25 µl of either whole blood or isolated cell suspensions was diluted in 20 ml of isotonic counting solution (Isoton® II, Counter Electronics, D-47705 Krefeld, Germany). And 200 µl of lysis solution (containing (g/l) KCN 3.3, sodium nitroprusside 1.1, quaternary ammonium salt 55; Zap-Oglobin®, Coulter Electronics) was added to the suspension of whole blood. One hundred µl of the lysis solution was added to the suspension of isolated blood and milk cells. Determination of number of leukocytes in the isolated counting solutions was applied by setting the instrument at 0.707 ma (milli Ampere) with the “dual threshold model”. The lower size threshold (TL) for whole blood samples was used at 3.86 µm, whereas the TL for isolated cells from blood and milk was applied at 4.98
μm. The number of cells with an upper size than the TL was applied for quantification of cells in the sample. The counting procedure was always performed in triplicate for each sample and the mean was calculated.

3. Results and discussion

PMN were characterized by their multi-lobed or sometimes picnotic dark-bluish stained nuclei (see figures 1, 2, 3 and 4). M morphologically had a large size, a round-vacuolated nucleus and contained whitish globules in their cytoplasm. And L was either large with a low nucleus to cytoplasm ratio and high blue stained nucleus or small with a high nucleus to cytoplasm ratio and a regular dark bluish stained nucleus. Epithelial cells were identified as large, polygonal and uniform stained-light bluish cells. The low amount of cells in milk from healthy cows (< $10^5$ cells/ml; Laevens et al., 1997), makes the identification of leukocytes more difficult than in high SCC milk (10$^9$ cells/ml during e.g., coliform mastitis; Dosogne et al., 1997). Nevertheless, the obstacle of cell differentiation unequivocally links to the inappropriate isolation procedure. For example, unwashed milk pellets would be indistinguishable (figure 1), affecting on counting procedure, which is based on particles and size, and overall PMN functional assay. For comparison, figure 1 with isolated cells after first step of centrifugation considerably differed from those after whole steps of isolation (figures 2, 3 and 5).

In isolation procedure of non-mastitis milk, it is almost impossible to yield 100% pure PMN. Due to the existence of a variety of cells (PMN, M, L and a small number of epithelial cells) in non-mastitis milk, the cellular source of mixed cells needed to be identified. This is special for the presence of the M in isolates, of which contribution to PMN functional assay would be crucial. This led us to develop a simple and rapid method to fractionate PMN from M to accurate assessment of their function. Figure 4 is the scanning electron micrograph of isolated PMN. The surface morphology of blood and milk PMN is distinguishable; milk PMN has less protruding pseudopods than that of blood. This might be resulted from diapedesis through blood/milk barrier and ingestion of milk fat globules and other particles (see figure 4).

Wide range of cell population in blood is also critical for identification, purification and eventually PMN functional assay. This is especially true while isolating blood PMN from mastitic animals who are sometimes neutropenic and the % of immature cells in circulation is high (figure 1). Experience revealed incomplete hypotonic lysis, during e.g., 30 seconds, hampers PMN functional assay. Since neutrophil function stay unchanged during 60 seconds exposure to the hypotonic lysis (Thorson et al., 1995), lysis duration of 60 seconds is advisable in order to avoid incomplete hypotonic lysis during isolation procedure. In blood cell identification procedure, we found a very well correlation between the % of immature neutrophils in the cells from whole blood and isolates (data not shown), indicating that isolated blood PMN are representative of whole blood PMN, thereby simplifying the interpretation of PMN functional assay.
Figure 1: Whole blood smear (left), immature neutrophil (arrow); milk cells after first step of centrifugation (right), which is almost indistinguishable.

Figure 2: Percoll isolation of macrophages (left; A and B) and PMN (right; C and D) form non-mastitis milk.
Isolation, differentiation and quantification of blood and milk neutrophils

Figure 3: Isolated PMN from normal cow blood (left), which is pure, and milk (right) PMN (B, C and D) macrophage (A) and lymphocyte (E).

Figure 4: Scanning electromicrograph of PMN isolated from blood (left) and milk (right). Protruding pseudopods needed for phagocytosis in PMN, which is distinguishable in blood and milk. The blood PMN has higher convulsed cell membrane that forms protruding pseudopods. This might be due to phagocytosis of milk fat globules and casein miscelles by milk PMN.
Figure 5: Isolated PMN from blood (right), in which the presence of immature neutrophil (arrow) is distinguishable and milk (left), in which phagocytosed *E. coli* (arrow) are visible. Samples were taken from cows suffering from *E. coli* mastitis.

In conclusion, the careful manipulation of blood and milk samples yields a sufficient amount of pure and functional PMN from blood and low-and-high SCC milk samples. This technique is simple, inexpensive and reproducible for further PMN functional assay.

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**References**

Chapter 2

Quantification of viability in blood and milk neutrophils and its relation with chemiluminescence

This chapter is based on:


Abstract

A flow cytometric technique was used to detect necrosis of bovine blood and milk polymorphonuclear leukocytes (PMN), applying propidium iodide (PI) exclusion method. This was confirmed with microscopic observation of PMN using trypan blue dye exclusion according to Colotta et al. (1992). Furthermore, the correlation between PMN viability and chemiluminescence (CL) was examined. Isolated blood and milk PMN was appropriately identifiable on FS-SS dot plot. We also found a significantly positive correlation between PMN viability and CL activity. Following an appropriate PMN isolation and under the optimal experimental conditions for measurement of blood and milk PMN viability defined in this study, flow cytometry is an accurate and reproducible technique for the rapid quantification of PMN survival in physiological and pathological conditions of dairy cows. This can effectively facilitate further PMN functional assay, hence boosting insight into the first line defense mechanism of the animal.
1. Introduction

A functional udder immune system depends on the existence of high quality Polymorphonuclear leukocytes (PMN) in milk, protecting the gland against invading pathogens (Burvenich et al., 1994; Kehrli et al., 2001). Investigation on PMN viability can provide suitable information about PMN quality and tissue damage. This is more special for milk PMN, which migrate to the apparently unsuitable environment. PMN life span might be affected by many physiological and pathological factors. Many cellular and acellular signaling pathways are available in blood and mammary tissue for the modulation or inhibition of PMN survival. Till now, little attention has been paid on the milk cell viability. Accordingly, the contribution of neutrophil enzymes (e.g., NADPH-oxidase activity) to the viability is critical (Mayer et al., 1989; Jankowski et al., 2001). This supports the assumption of the existence of a good correlation between PMN viability and CL. To obtain a better insight into the effects of (patho)physiology of mammary gland on non-specific defense mechanisms of the udder, assessment of viability of blood and milk PMN can be pivotal. Milk PMN viability assessment could also be an index for the detection of mastitis.

2. Materials and methods

Animals
Clinically healthy Holstein-Friesian cows (first to third lactation; day 21±6 to 210±36 of lactation; n = 66) from the Ghent University dairy farm (Biocentrum Agri-Vet Melle, Belgium) were selected for blood and milk sampling. Cisternal quarter milk samples (500ml) were aseptically collected, using a sterile teat cannula (Vangroenweghe et al., 2000). Milk samples were stored on ice until cell isolation. Following milk sampling, blood samples (80 ml) were aseptically collected from the external jugular vein into evacuated tubes (Laboratoire EGA, Nogent-le Roi, France) containing 125 I.U. heparin.

Blood and milk PMN isolation
Blood and milk PMN isolations were performed as described in chapter 1, yielding 98±1% and 79±8% pure PMN for blood and milk, respectively. The isolated PMN were subsequently quantified and differentiated. The isolated blood and milk PMN were then preserved in Dulbecco's PBS (DPBS) (Gibco BRL, Life Technologies Inc., MD, USA) supplemented with gelatin (0.5mg/ml, Merck, Darmstadt, Germany). PMN concentration was adjusted between $1 \times 10^6$ and $5 \times 10^6$ cells/ml and samples were kept at 4°C.

PMN Viability assessment
The viability of isolated milk and blood PMN was assessed using flow cytometry (FACSScan, Becton Dickinson Immunocytometry Systems, San José, CA, USA). After addition of 10 µl propidium iodide (PI) (50 g/ml PBS; Sigma Chemicals, St. Louis, Mo, USA) to 490 µl of isolated cells ($1.10^6$ cells/ml DPBS, final concentration), the red fluorescence of PMN was measured. Two criteria were chosen for the correct gating of PMN in the Forward Scatter (FS)-Side Scatter (SS) dot plot: 1) flow cytometric analysis of percoll-purified PMN (Mehrzad et al., unpublished; confirmed by light microscopy); a linear positive correlation was found between % PMN diagnosed by light microscopy and the PMN gated in the FS-SS dot plot ($R = 0.96; P < 0.001; n = 66$); 2) labeling of PMN with a specific monoclonal antibody.
(mAb) against bovine granulocytes (cell line CH 138A, VMRD, Inc., Pullman, WA, USA) and a secondary fluorescein isothiocyanate (FITC)-labeled antibody (Serotec Ltd., Oxford, England) (Davis et al., 1987). Red fluorescence of PI-incubated cells was registered at 650 nm using the 488 nm excitation wavelength. In the red fluorescence histogram, a gate (M1) was set to discriminate between living and dead cells, with the lowest limit higher than auto fluorescence. All cells within M1 gate were considered as dead. The threshold value for discrimination between live and dead cells was determined in isolated cells without PI addition, so that in samples without PI, 100 % cells were below the threshold value. Percent of cells with a lower value than the threshold was used for the calculation of the percent viability.

**Relation between PMN viability and CL**

To investigate the eventual relationship between milk PMN viability and CL, the flow cytometric viability values were simultaneously compared with the PMN CL (see chapter 3 for CL assay). The Spearman correlation coefficient was used to assess the correlation between PMN viability and CL. It was further tested whether this correlation coefficient was significantly different from 0.

**3. Results and discussion**

The coefficient of variation (C.V) for the viability of PMN in isolated cells had an average of 4.3±0.6 % for milk and 1.7±0.5 % for blood. This method corresponded to the microscopic trypan blue exclusion method. Flow cytometry was more accurate than microscopy, with a C.V. of roughly 5 % (10000 cells counted) compared to a C.V. of about 20 % for the microscopical method (200 cells counted). Figure 1 demonstrates representative results of PMN viability analysis.

This method of milk PMN viability is fast and accurate, provided the isolation procedure is appropriate. In percoll isolated milk PMN, we found that the highest quality of PMN are precipitated at the bottom layer.

The use of mAb labeled PMN is important in particularly milk PMN during different stages of lactation and mastitis. The labeled PMN are effective in diagnosis because they accumulate at the site of inflammation. PMN separation techniques should be fast and precise to avoid any potential damage to PMN (i.e., contamination of some cellular contaminants or inappropriate hypotonic lysis). There is little information on the optimal time for the hypotonic lysis exposure, during which the PMN viability is compromised. Our study addressed that issue and revealed little or no side effect on PMN viability after exposing them with two consecutive times of 50 seconds to hypotonic lysis (data not shown). Prolonged incubation of PMN at 37°C declined PMN viability and CL, this is more important for milk PMN. This should be considered while studying PMN functional assay for a long time at 37°C incubation. Experience suggests that low temperature (e.g., 4 °C) delays *in vitro* PMN necrosis. This should be implemented from milk sampling to immediately before viability assay.
Quantification of viability in blood and milk neutrophils and its relation with chemiluminescence

Figure 1: Representative results of flow cytometric analysis of isolated bovine milk PMN gated in the FS-SS dot plot (upper panel). Green fluorescence of PMN labeled with a monoclonal antibody specific against bovine PMN and with a secondary FITC-labeled antibody (middle panel). Red fluorescence of propidium iodide-incubated PMN selectively gated in the FS-SS dot plot (lower panel). For the PMN viability quantification, gate M1 is applied to determine the percentage of necrotic PMN (i.e., PI-stained PMN).
There was a significant positive correlation between PMN viability and CL (figure 2). ($\rho = 0.94; p = 0.0001$). The mechanism for survival of PMN and its relation with CL is far from conclusive. Based on studies (Mayer et al., 1989; Jankowski et al., 2001) the contribution of PMN NADPH-oxidase activity to viability appears critical, contributing to phagosomal and cytosolic pH homeostasis. Many more mechanisms such as caspase activity (Fadeel et al., 1998; Akgul et al., 2001) are pivotal for promoting PMN necrosis. The viability assessment gives some idea about the cytochemical, cytoskeletal and phagocytosis status of the PMN. Therefore, blood and milk PMN viability examination would be very important approach of mimicking first line defense mechanism of high yielding dairy cows.

In summary, flow cytometry is an accurate and reproducible technique for the rapid quantification of PMN survival in normal and mastitis conditions of dairy cows. This can effectively facilitate further PMN functional assay, hence boosting insight into the first line defense mechanism in the high yielding dairy cows.

Figure 2: The Spearman correlation coefficient between milk PMN CL and viability ($n = 66$). Very significant correlation between PMN CL and their viability ($\rho = 0.94; p = 0.0001; n = 66$).
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References


Chapter 3

A comparative study of bovine blood and milk neutrophils functions with luminol-dependent chemiluminescence

This chapter is based on:

J. Mehrzad, H. Dosogne, F. Vangroenweghe and C. Burvenich

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Abstract

In this study, a technique was developed for the chemiluminescence (CL) measurement of bovine milk polymorphonuclear leukocytes (PMN). In the first study, the effects of cell number and the concentration of phorbol-12-myristate-13-acetate (PMA), luminol, latex bead particles, dimethyl sulphoxide (DMSO) and gelatin on the luminol-dependent cellular CL (LDCL) response were assessed with healthy cows in different stages of lactation. In the second study, the LDCL and in vitro bactericidal activity of blood and milk PMN towards \textit{Staphylococcus aureus} (\textit{S. aureus}) was investigated. In general, the CL activity of blood PMN was consistently higher than that of milk PMN. We found that 1) the optimal cell density in blood and milk cells for maximal LDCL response ranged from $1.5 \times 10^6$ to $5 \times 10^6$ cells / ml; 2) the optimal concentrations of PMA, latex beads and luminol for maximal LDCL response were 100 to 200 ng / ml, 500 particles / PMN and 0.1mmol/L, respectively. The concentrations of DMSO between 0.5% and 1% (v / v) did not significantly affect the maximal CL response of PMN. Gelatin concentrations between 0.1 to 0.5 mg / ml had no effect on the LDCL of PMN. In addition, the LDCL of PMN was significantly correlated with bactericidal activity towards \textit{S. aureus} ($r = 0.78$, P < 0.001 for blood PMN and $r = 0.66$, P < 0.01 for milk PMN). Under the optimal experimental conditions for measurement of CL produced by bovine blood and milk PMN defined in this study, LDCL assay is an accurate and reproducible technique for the rapid quantification of PMN bactericidal activity in physiological and pathological conditions of high yielding dairy cows.
1. Introduction

In high yielding dairy cows, early lactation-related infectious diseases such as environmental mastitis and metritis are detrimental for the bovine industry and blood polymorphonuclear leukocytes (PMN) are of major importance in the defense against these diseases (Burvenich et al., 1994; Paape et al., 1996). Post-diapedetic PMN in milk have been considered as rather ineffective cells. However, several investigations have demonstrated a remarkable reactive oxidant species (ROS) produced by milk PMN (Weber et al., 1983; Dulin et al., 1988; Piccinini et al., 1999; Mehrzad et al., 2001a). Because blood and milk PMN chemiluminescence (CL) is positively correlated to milk PMN (Mehrzad et al., 2001b) and because of the instant exposure of milk PMN to pathogens at infected sites, application of the CL technique for bactericidal activity of PMN could lead to estimation of the cow susceptibility to the crucially lactation related disease: mastitis.

During the respiratory burst activity of PMN, ROS such as \( \text{H}_2\text{O}_2 \), \( \text{O}_2^- \), \( \text{OH}^- \), \( \text{1O}_2 \) and \( \text{HOCl} \) are generated. These can be quantified by means of different techniques such as the cytochrome \( c \) reduction test, flow cytometry (Salgar et al., 1991) and the scopoletine test (Root et al., 1975). However, the most widely used technique to estimate the respiratory burst activity of PMN is the CL assay, which has first been described by Allen et al. (1972). This sensitive CL results from reactions of ROS with luminescence agents, requires both an active NADPH-oxidase, the key-enzyme of the respiratory burst, and an active myeloperoxidase (MPO), the enzyme of the azurophilic granules of PMN (Webb et al., 1974).

Developing an accurate and reproducible method to quantify the oxidative metabolism of blood and milk PMN using the luminol-dependent CL (LDCL) technique could, inexpensively, give us a conclusive idea about PMN bactericidal efficiency. Today sophisticated analytical apparatus are available for the measurement of CL of blood PMN, which has been amply demonstrated either in human or less frequently in bovine, but poorly and insufficiently investigated upon milk PMN, nor compared with blood PMN in physiological conditions. Due to the existence of a variety of cells in isolated cells from normal milk, CL-enhancer and activator agents, DMSO and cell aggregation preventing agents, such as gelatin, are required. It is not clear how all these factors influence the LDCL assay of blood and milk PMN. To obtain unequivocal results from the LDCL assay, some important considerations should be taken into account while using this elegant technique. In addition, LDCL resulting mainly from intracellular ROS production (Dahlgren et al., 1983; Briheim et al., 1984) could potentially and ultimately lead to killing of endocytosed microorganisms. To demonstrate the contribution of blood and milk PMN towards the killing of invading microorganisms in the mammary gland, the relationship between LDCL response of PMN and intracellular killing of \( \text{S. aureus} \) has also been investigated.

A detailed description of the optimised measurement conditions of blood and milk PMN in high yielding dairy cows is presented in this study.
2. Materials and Methods

Animals and experimental procedures
Forty-eight healthy Holstein-Friesian cows from the Ghent University dairy farm (Biocentrum Agri-Vet, Melle, Belgium) were selected on the basis of 2 consecutive bacteriologically negative milk samples and somatic cell counts (SCC) below 2.10^5 cells/ml milk per individual quarter. In the first study, three different stages of lactation were investigated: early lactation cows (3 to 31 d after calving, n = 10) mid lactation cows (150 to 210 d after calving, n = 10) and late lactation cows (220 to 300 d after calving, n = 10). In the second study, 18 cows from the same farm in their 2nd to 3rd lactation were selected: early lactation cows (3 to 20 d after calving, n = 6) mid lactation cows (155 to 220 d after calving, n = 6) and late lactation cows (235 to 300 d after calving, n = 6) for the bactericidal assay. Blood samples were aseptically collected from the external jugular vein into evacuated tubes (Laboratoire EGA, Nogent-le Roi, France) containing 125 I.U. heparin. Cisternal milk samples were aseptically collected after cleaning and disinfection of the teats using a sterile teat cannula according to Vangroenweghe et al. (2001) and stored on ice until processed. The mammary gland did not stimulated prior to milk collection.

Preparation of PMN from blood and milk
All materials and reagents used for the isolation of blood and milk PMN were sterile. According to Carlson et al. (1973) isolation of PMN from peripheral blood was performed, and erythrocytes lysis was done using hypotonic agent. PMN was counted using an electronic particle counter (Coulter counter Z2, Coulter Electronics Ltd., Luton., UK). Briefly, 25 µl of isolated cell suspensions were diluted in 20 ml isotonic counting solution (Isoton® II, Counter Electronics, D-47705 Krefeld, Germany). Hundred µl lyse solution (containing (g/l) KCN 3.3, sodium nitroprusside 1.1, quaternary ammonium salt 55; Zap-Oglobin® ; Coulter Electronics) were added to the suspension of isolated blood cells in order to lyse remaining erythrocytes, whereas no lysis solution was used for the isolated milk cells. The instrument current was set at 0.707 mA (milli Ampere) with the dual counting threshold model. The lower size threshold (Tl) for isolated cells from blood and milk was set at 4.98 µm. The number of cells with an upper size than the Tl was applied for quantification of cells in the sample. Counting procedures were performed in triplicate.

The viability of isolated PMN were determined by means of flow cytometry (FACSScan, Becton Dickinson Immunocytometry Systems, San José, CA, USA), using propidium iodide exclusion (Mehrzad et al., 2001b). % PMN in blood and milk was assessed as described previously (Mehrzad et al., 2001b). The cell suspension was adjusted to different concentrations in Dulbecco's PBS (DPBS) (Gibco BRL, Life Technologies Inc., MD, USA) supplemented without or with gelatin (0.5 mg/ ml, Merck, Darmstadt, Germany).

Initial volumes of 2 L milk were processed using a high-capacity centrifuge (RC – 3BP, Sorvall, Newtown, CT, USA) after 60 % v/v dilution with cold phosphate-buffered saline (PBS; 0.01 mol / L phosphate buffer (KH2PO4-Na2HPO4) – 0.15 mol / L NaCl, pH = 7.2). Fat was carefully removed after the first centrifugation (600 x g, 15 min, 4°C) and the pellet was washed twice at 300 x g for 10 min and 200 x g for 15 min at 4°C, in cold PBS. The cells were finally resuspended in DPBS supplemented without or with gelatin (0.5 mg / ml). After counting the isolated milk cells, determination of viability and % PMN, the cells were resuspended to different
concentrations in DPBS supplemented with or without gelatin, and finally assessed for LDCL assay.

**Identification of blood and milk cells**

Plasma was added to the isolated cells in a 1:1 ratio to prevent lysis during preparation of the smears. Differential cell counts on the isolates were performed on eosin-Giemsa-stained smears (Hemacolor® Merk Diagnostics, Darmstadt, Germany), using light microscopy. Identification of the cells in blood was based on morphological characteristics as described by Hayhoe and Flemans, (1969). Milk cell differentiation was based on morphological characteristics as described by McDonald and Anderson, (1981), with some modification as recently explained (Mehrzad et al., 2001a; b). PMN (mature and immature), monocytes / macrophages, lymphocytes and epithelial cells (only in milk) were identified on 200 cells per slide.

**Luminol-dependent chemiluminescence (LDCL) assay in blood and milk PMN**

LDCL assays of blood and milk PMN measured upon stimulation with PMA (phorbol 12-myristate 13-acetate) and latex beads. Briefly, the LDCL was performed using computerized programmable luminometer (type LB96P; EG&G Berthold, D-75312 Bad Wildbad, Germany) at 37 °C. A total volume of 200 µl was prepared for the assay. Immediately after addition of PMA (at a final concentration of 200 ng / ml) and luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione at a final concentration of 0.3 mmol/L, Sigma) to PMN with variable concentration into the wells of round-bottomed-transparent white microtiterplates (Berthold, D-75312 Bad Wildbad, Germany) LDCL was measured. The area under the curve (AUC) was calculated for registered impulse rate (counts / min) over the entire measurement period of 30 min. The CL response was corrected for $10^3$ viable PMN in each sample. The following formula was used to perform the corrections:

$$CL_{PMN} = \frac{CI_{isolated\ cells} \times 10^3}{N \times \% \ PMN \times \% \ V}$$

where $Cl$ = mean RLU / s, $N$ = total number of cells per well, $\% \ PMN$ = total percentage of PMN in isolated cells, $\% \ V$ = percentage viable PMN.

The LDCL of blood PMN was calculated with the same formula as for milk PMN applying the corrections described by Heyneman et al. (1990) and recently modified according to our previous study (Mehrzad et al., 2001a; b) for interference of eosinophils.

With regard to the CL measurement, all assays were carried out at pH of 7.2 and at 37°C.

**Preparation of reagents**

**PMN concentration**

The effect of different concentrations of both blood and milk PMN on LDCL was investigated following stimulation with 200 ng / ml PMA and 0.1 mmol/L luminol. Concentrations ranging from 0.25 to $5.5 \times 10^6$ PMN /ml were examined.

**PMA and latex beads particle concentrations**

A stock solution of 200 µg / ml PMA was prepared in DMSO (dimethyl sulphoxide; Sigma) and stored at -20 °C. PMA was tested at concentrations of 0, 25, 50, 100, 200
and 500 ng / ml for both blood and milk PMN with a final concentration of $2 \times 10^6$ PMN / ml and 0.1 mmol/L luminol.

Latex beads (polystyrene 0.76 µm diameter, $4.10^{11}$ particles / ml; Sigma) was evaluated at final concentrations of 0, 250, 500, 1000, 2000 and 2500 particles / PMN with the same concentrations of PMN and luminol as with PMA. Both for PMA and latex-stimulated LDCL, mean CL index (MCLI) was calculated using $\text{MCLI} = (\text{SCL} – \text{NSCL}) / \text{NSCL} \times 100$, where SCL is Stimulated CL (AUC of 1000 viable PMN) and NSCL is non-stimulated CL (AUC of 1000 viable PMN).

**Luminol concentration**

A luminol stock solution of 100 mmol/L was prepared in DMSO and stored at -20 °C. The effect of six different concentrations of luminol on both blood and milk PMN CL was tested: 0.01, 0.1, 1, 5, 10 and 20 mmol/L, using stimulation of $2 \times 10^6$ PMN / ml with 200 ng / ml of PMA.

**DMSO concentration**

Both blood and milk PMN ($2 \times 10^6$ PMN / ml) were incubated at 37°C with DMSO at final concentrations from 0.5 to 10% for 30 min. Subsequently, PMN were stimulated with 200 ng / ml PMA in the presence of 0.1 mmol/L luminol for the LDCL assay. Finally the AUC of 1000 viable PMN was quantified.

**Gelatin**

Gelatin (Merk; Germany) was used to prevent PMN agglutination. Both blood and milk PMN ($2 \times 10^6$ PMN / ml) were incubated at 37°C with gelatin at final concentrations from 0.1 to 0.5 mg / ml for 30 min. Subsequently, PMN were stimulated with 200 ng / ml PMA in the presence of 0.1 mmol/L luminol for LDCL quantification.

**Variability**

The coefficient of variation (CV) for blood and milk PMN LDCL assay of the same sample was measured in 30 cows. Day-to day variation of this assay was also tested for blood and milk PMN in 6 cows for 5 consecutive days during three different stages of lactation.

**Relation between bactericidal activity and LDCL of PMN**

In the second study, in order to investigate relation between CL and bactericidal activity of blood and milk PMN, PMN LDCL was simultaneously compared with the bactericidal activity of *S. aureus* Newbould 305 (Naidu et al., 1975). The killing of bacteria was monitored by a bactericidal assay using sample cultivation, accordingly, (Williams et al., 1985; Barrio et al., 200). Briefly, 100 µl live bacteria ($5.10^7$ / ml) were added to 500 µl viable PMN isolated from milk ($5.10^6$ / ml). After dilution and plating out the mixture in duplicate onto Columbia sheep blood agar (Biokar Diagnostic, Beauvois, France), the plates were incubated overnight at 37°C and colony counts were performed. Results from the bacteriological assay are expressed as the percentage of killed (% killing) *S. aureus* compared to the initially administrated amount of bacteria.

**Statistical analyses**

For the statistical analyses the Statistix program package (version 4.1, Analytical Software, Tallahassee, FL, USA) was used. A two-way analysis of variance was
performed following a Bartlett’s test of equal variances. The time of sampling was a fixed factor, cows were a randomized factor, and their interaction term was the error term. Comparisons of means were performed by the least significant differences (LSD) test. Significant differences were determined at $P < 0.05^*$, $0.01^{**}$ and $0.001^{***}$.

The following linear model was used to analyse the correlation between PMN LDCL and bactericidal efficiency.

$$Y = ax + b + c$$

Where $Y = \%$ of killing, $a = \text{regression coefficient}$, $X = \text{CL of milk PMN}$, $b = \text{intercept}$ and $c = \text{error term}$.

3. Results

Optimal PMN concentration

Effect of concentrations of PMN on LDCL response are presented in Figure 1. In all stages of lactation, the CL activity of blood PMN was about 3.5 times higher than that of milk PMN, irrespective of the PMN concentration. Because the PMA – induced LDCL of PMN both in blood and milk was lower in early lactation than in mid and late lactation, the optimal concentration of PMN isolated from blood ranged from $1.5 \times 10^6$ to $3.5 \times 10^6$, $0.5$ to $2.5 \times 10^6$ and $1.5$ to $2.5 \times 10^6$ cells / ml in early, mid-and late lactation cows, respectively (Figure 1a). The optimal concentration of PMN isolated from milk ranged from $1.5 \times 10^6$ to $3.5 \times 10^6$, $0.25$ to $4 \times 10^6$ and $0.5$ to $4 \times 10^6$ cells / ml in early, mid-and late lactation cows, respectively (Figure 1b).

PMA concentration

Although the PMA-stimulated LDCL of blood PMN was higher than their milk counterparts, the optimal concentrations of PMA for a maximal LDCL response were between $100$ to $500$ ng / ml for blood PMN for all stages of lactation. At this range of optimal PMN concentration, no significant increase of maximal LDCL responses was observed by increasing the concentration of PMA (Figure 2a). Unlike blood PMN, the optimal PMA concentrations for maximal LDCL response were between $100$ to $200$ ng / ml for milk PMN for all stages of lactation. By increasing the PMA concentration to $500$ ng / ml, the maximal LDCL response was decreased (Figure 2b).

Latex beads particles

Regardless of the differences between latex-stimulated blood and milk PMN LDCL and the differences between different stages of lactation, the optimal concentrations of latex beads for maximal LDCL responses of blood PMN were $500$ particles / PMN (Figure 3a). The concentrations of latex beads for maximal LDCL responses of milk PMN were also $500$ particles / PMN (Figure 3b). Interestingly, both in blood and milk PMN as well as in all stages of lactation, the maximal CLI declined by increasing the latex beads beyond the $500$ particles / PMN (Figure 3a; b).
Luminol
The luminol concentration was critical for maximal CL of both blood and milk PMN. In blood, at all stages of lactation, the CL response increased in dose-dependent manner by increasing the concentration of luminol from 0.01 to 0.1 mmol/L, whereas beyond 0.1 mmol/L the CL response decreased (Figure 4a). Similarly, milk PMN CL revealed the same pattern as observed in blood but with less pronounced effect (Figure 4b). Both in blood and in milk PMN a final concentration of 0.1 mmol/L resulted in a maximal CL response (Figure 4a; b).

DMSO
No significant effects were observed on blood LDCL by adding concentrations of DMSO from 0 to 1% (v / v). The LDCL response decreased, in a dose dependent manner, at DMSO concentrations of 2.5% (v / v) and higher (Figure 5a). In milk PMN, DMSO concentrations of 0 and 1% (v / v) did not have any effect on the LDCL response, whereas DMSO concentrations of 2.5% (v / v) and higher resulted in a dose-dependent decrease of the LDCL response (Figure 5b).

Gelatin
Both in blood and milk PMN, gelatin concentrations from 0.1 to 0.5% did not have any effect on the LDCL response (data not shown).

Variability
The average CV for blood and milk PMN LDCL measurements of the same sample was 4.3 ± 1.1 and 8.8 ± 2.6 respectively. The day-to-day variation of LDCL assay within a cow was tested in 6 cows, yielding 3.5 ± 0.8 %, 2.6 ± 0.3 % and 2.8 ± 0.4 % for blood PMN during early, mid-and late lactation respectively, whereas the day-to-day variation was 11.5 ± 3.5, 5.2 ± 0.7 and 5.7 ± 1, respectively for milk PMN.

Relation between bactericidal activity and PMN LDCL
The percentage killed S. aureus by milk PMNL was lower than blood PMN in all stages of lactation. The percentage killed S. aureus by either blood or milk PMN was lower in early lactation than in mid-and late lactation. During early, mid-and late lactation, blood PMN bactericidal activity was 27 ± 5 %, 71 ± 9 % and 69 ± 8 %, whereas in milk PMN it was 13 ± 5 %, 54 ± 7 % and 52 ± 6 %, respectively. A significant positive correlation was found between PMA-induced LDCL of PMN and their bactericidal activity: r = 0.78, P < 0.001 for blood PMN (Figure 6a) and r = 0.66, P < 0.01 for milk PMN (Figure 6b).

4. Discussion
Accordingly (Allen et al., 1972; 1986), in this study the sensitive, accurate and widely used CL assay was concurrently applied in bovine blood and milk PMN and so investigated their bactericidal efficiency. Despite substantial PMN ROS production, there remains one remarkable difference between blood and milk PMN as well as between periods of lactation: the intensity of ROS production (Mehrzad et al., 2001b). Most recently liquid scintillation counter has been applied to measure ROS production of bovine blood PMN, appeared difficult and required intensively manual adjustments, thereby time-inefficient (Mehrzad et al., 2000). Contrary, the
luminometer used in the current study has been specifically designed, no pre-manually adjustment requires and the technique would also be fairly applicable to non-mastitic milk PMN functional assay. To quantify the LDCL, as recently demonstrated the obtained data are preferentially expressed as integrated CL response, covering the entire 30-minutes measurement period (Mehrzad et al., 2000).

The isolation procedure of PMN from blood resulted in a yield of granulocytes (PMN + eosinophils) >95 % with predominantly PMN (>85%) and a viability of 98 % in all stages of lactation. Although the isolation of PMN from non-mastitic milk is rather difficult, a yield of >80 % PMN (no eosinophils) with a variable viability was observed in different stages of lactation. According to the formula used for calculating of CL and unlikely contribution of other milk cells to the LDCL, unequivocal results of milk PMN CL are likely. In blood cells, erythrocytes and free hemoglobin reduce LDCL (Glette et al., 1982) by quenching the blue light emitted by ROS-related oxidation of luminol by-products. This problem was resolved by hypotonic isolation procedure. In non-mastitic milk PMN, the question of appearance of either erythrocytes or free hemoglobin is fairly irrelevant. However, the existence of a variety of cells in milk, particularly macrophages, could partly be problematic for the LDCL assay of milk PMN, because milk macrophages are devoid of any MPO-H2O2 system (Hallén Sandgren et al., 1991; Mehrzad et al., 2001b) and consequently produce no LDCL. Also lucigenin-dependent CL by milk macrophages is insignificant (Hallén Sandgren et al., 1991), so the contribution of macrophages to our study is negligible. These previous findings allowed us to apply the formula to quantify, unequivocally, LDCL of milk PMN.

In both blood and milk, extremely high and low PMN concentrations generated a suboptimal CL response at constant sample volumes of 200 µl. Suboptimal LDCL responses at high PMN concentration can be explained by fewer light counts due partly to the potential cell aggregation, resulting in quenching of light. More pronounced adverse effects of high PMN concentration samples on LDCL may also result from higher self-absorption of light. In low PMN number samples, suboptimal CL could be due to the fact that the liquid is too weak to be detected by the instrument. Nonetheless, if PMN concentration range between 1.5 \times 10^6 and 5 \times 10^6 cells/ml, for both blood and milk, maximal LDCL responses are observed, revealing high selective intensity of luminometer application to obtain the maximal LDCL response.

LDCL was stimulated in a phagocytosis-independent manner with PMA, a known-potent protein kinase-C agonist and initiator of NADPH-oxidase activity (White and Esensen., 1974; DeChatelet et al., 1976; Karlson et al., 2000), and in a phagocytosis-dependent manner with latex beads. Latex beads activate PMN by a mechanism independent of changes in intracellular calcium involving protein kinase-C (Hallet et al., 1983).

In blood PMN, both PMA and latex beads always exhibited stimulation for ROS production. Compared to PMA, opsonized zymosan particles have been reported to induce more pronounced PMN CL response (Hallet et al., 1983; Cooke and Hallett, 1985). Phagocytosis of zymosan particles, which exerts a similar effect on CL activity as the latex particles used in our study, induces a strong degranulation and release of primary as well as of secondary granules, even indirectly revealing involvement of Protein kinase-C (Hallet et al., 1983). Milk PMN showed the same pattern towards PMA as well as latex beads but the intensity was lower than blood PMN. This is in
agreement with previous studies (Duline et al., 1988; Mehrzad et al., 2001b). In addition, the CL response of milk PMN upon latex stimulation was much stronger than PMA stimulation, whereas the responses were similar in blood PMN. It is not surprising that CLI increased by increasing PMA concentrations, as reported for PMA concentrations of up to 1 µg/ml (Westrick et al., 1980), quoting a wide range of suitable PMA concentrations for the LDCL assay. The decreased CLI at concentrations above 500 latex particles / PMN could be attributed to scattering of the light photons by the latex beads, resulting in a decreased detection efficiency. To minimize this effect, latex beads concentrations should be below 500 particles / PMN. The lower values of latex-induced CLI during early lactation reveals less phagocytosis-induced oxidative burst activity compared to the mid- and late lactation. PMA-induced CLI was also lower during early lactation as in other studies (32). Diminished oxidative burst activity of PMN during early lactation is in agreement with other studies (Kehrli et al., 1989; Hoeben et al., 2000, Mehrzad et al., 2001b). Together, these results suggest that in early lactation, both the mechanism for ROS production itself and the pathway from phagocytosis to ROS production are impaired. Therefore, phagocytosis-induced and/or non-induced CL activity of PMN could be a feasible estimation of PMN bactericidal capacity.

The energy content of non-enhanced light photons produced by PMN is the detection limit of any luminometer. In this study luminol was used to amplify the light photons. This molecule directly penetrates into the PMN and can enhance intra-cellular light and/or ROS production, yielding an electronically excited aminophtallate (Faulkner et al., 1993). In our study, a maximal CL response was obtained using 0.1mmol/L luminol with both blood and milk PMN. Beyond this concentration, the CL response was inhibited, in accordance to other study (Allred et al., 1980), observing the highest CL response at 0.1 mmol/L and lower responses at higher concentrations. The reduced effect of higher luminol concentrations could be attributed to a cytotoxic effect of luminol. Indeed, luminol has been shown to inhibit NADPH-oxidase activity in a dose-dependent manner (Fäldt et al., 1999).

One of the most commonly used solvents for PMA and luminol is DMSO. Despite the known cytotoxic effects of DMSO, no adverse effects on LDCL were observed at the concentration of 1% (v/v) used in this study, indicating safety and applicability of low DMSO concentrations towards bovine blood and milk PMN in functional assays. This finding is also in agreement with a previous study in human PMN (Heinle et al., 1995). In contrast, at concentrations higher than 1% (v/v), adverse effects of DMSO on LDCL of both blood and milk PMN were not surprising. Cytotoxic effects of the cell-permeable DMSO on PMN exhibit depletion of cytoplasmic granules or degranulation, thereby decreasing the LDCL response. A higher percentage of dead PMN further substantiates the cytotoxic effect of high DMSO concentration. Inhibitory effects of DMSO on PMN LDCL were less apparent with shorter incubation time, ≤30 minutes. This is consistent with degranulation effects of DMSO as the second LDCL peak has been proposed to arise from intracellular ROS production in human PMN (Briheim et al., 1984). Apparently effects of DMSO were more pronounced in milk than in blood PMN. Finally, it can be concluded that DMSO concentrations used in our study have no inhibitory effects on LDCL of blood or milk PMN.
Another practical issue in measuring PMN LDCL is PMN aggregation. To prevent any potential PMN aggregation and to minimize light quenching, the use of gelatin for the LDCL assay was evaluated. Gelatin is applicable for both milk and blood PMN. No significant effects of gelatin on the LDCL assay were observed. This suggests that no oxidation-reduction reactions occurred between gelatin and ROS during the LDCL assay. Therefore, gelatin is proposed as an appropriate measure to prevent cell aggregation during the CL assay.

Compared to blood PMN LDCL assay, the higher CV in milk PMN LDCL assay was observed. According to the formula for calculating CL, cell counting, cell differentiation and viability quantification unquestionably influence on the CV of PMN LDCL. To minimize the CV of PMN LDCL, cell counting, differentiation and viability were appropriately assessed. Nevertheless, the higher CV of milk PMN LDCL possibly resulted from differences among milk PMN in uptake of fat globules and casein (Paape et al., 1977). The higher variability in LDCL during early lactation would be due in part to inevitable hormonal and metabolic changes, resulting in PMN LDCL alteration (Hoeben et al., 2000; Mehrzad et al., 2001b). These remarkable changes require further studies.

Based on our results, LDCL seems to be an appropriate estimation of PMN bactericidal efficiency. Endocytosed bacteria are most efficiently killed by a complex cascade of oxidation-reduction reactions taking place inside phagolysosome, yielding the most potent bactericidal ROS, HOCL (Fantone et al., 1982), which can be measured by LDCL assay. In comparison to the bactericidal assay, LDCL is more time and cost-efficient and is in particular applicable for high-throughput measurements. Despite the less efficient towards killing of endocytosed bacteria and ROS production capacity by PMN during early lactation, a strong positive correlation was obtained between LDCL and bactericidal capacity. Remarkably, in each lactation stage, both ROS production and bactericidal capacity in milk PMN were lower than blood PMN. One of the primary explanations for this observation is the presence of fat globules in milk which are constantly ingested by milk PMN (Paape et al., 1977), resulting in a decreased phagocytic capacity of milk PMN, compared to blood PMN. Therefore, latex-induced LDCL is concluded to be an appropriate indicator for phagocytosis-induced ROS production by blood and milk PMN.

Efficiency of bactericidal activity is reflected by the kinetics of ROS generation: normally biphasic, with a first low-intensity and short-lasting phase and a second high-intensity and long-lasting phase as well as the second phase is absent in blood in early lactation (Mehrzad et al., 2001b). This could explain the less effective bactericidal capacity during the early post-partum period. In addition, the second phase was never observed in milk PMN either, which could also explain their impaired bactericidal capacity. In vitro PMN LDCL responses thereby reflect the bactericidal capacity of PMN. Further studies are in progress to explain the impaired bactericidal capacity of bovine PMN around parturition and during early lactation.

The optimal experimental conditions described for bovine blood and milk PMN LDCL enable application of the LDCL assay as a rapid, accurate and reproducible method for evaluating PMN bactericidal activity in physiological and pathological conditions of high yielding dairy cows. These advantages, therefore, make the LDCL assay an elegant method to explore the first line defense mechanism of cows.
Acknowledgements

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References


Figure 1. Effect of different concentrations of PMN on the luminol-dependent cellular CL of blood (a) and milk (b) PMN upon stimulation with 200 ng / ml of PMA in different stages of lactation. Values are expressed as the area under the curve (AUC) of RLU / S of $10^3$ viable PMN during 30 minutes. Values are means ± SEM of 10 cows. Significance of the difference between different concentrations of cells is indicated with asterisks (*: $P < 0.05$).
Figure 2. Effect of different concentrations of PMA on the luminol-dependent cellular CL of blood (a) and milk (b) PMN in different stages of lactation. Values are expressed as mean CL index which is the calculation of [PMA stimulated CL (AUC of RLU / S of 10^3 viable PMN during 30 minutes) – non-stimulated CL (AUC of RLU / S of 10^3 viable PMN during 30 minutes)] / non-stimulated CL. Values are means ± SEM of 10 cows. Significance of the difference between different concentrations of PMA is indicated with asterisks (**: p < 0.01).
Figure 3. Effect of different concentrations of latex particles on the luminol-dependent cellular CL of blood (a) and milk (b) PMN in different stages of lactation. Values are expressed as mean CL index which is the calculation of [latex stimulated CL (AUC of RLU / S of $10^3$ viable PMN during 30 minutes) – non-stimulated CL (AUC of RLU / S of $10^3$ viable PMN during 30 minutes)] / non-stimulated CL. Values are means ± SEM of 10 cows. Significance of the difference between different concentrations of latex is indicated with asterisks (**: $p < 0.01$).
Figure 4. Effect of different concentrations of luminol on the luminol-dependent cellular CL of blood (a) and milk (b) PMN in different stages of lactation after stimulation with 200 ng / ml of PMA. Values are expressed as the area under the curve (AUC) of RLU / S of 10^3 viable PMN during 30 minutes. Values are means ± SEM of 10 cows. Significance of the difference between different concentrations of cells is indicated with asterisks (**: P < 0.01; ***: p < 0.001).
Figure 5. Effect of different concentrations of DMSO, after incubation for 30 minutes at 37°C, on the luminol-dependent cellular CL of blood (a) and milk (b) PMN in different stages of lactation. Values are expressed as the area under the curve (AUC) of RLU / S of $10^3$ viable PMN during 30 minutes. Values are means ± SEM of 10 cows. Significance of the difference between different concentrations of cells is indicated with asterisks (**: P < 0.01; ***: p < 0.001).
Figure 6. Correlation between PMA induced LDCL of blood PMN (a) and milk PMN (b) and their effectiveness towards killing of *S. aureus*. E: early lactation period; L, M: late and mid lactation periods.
Part 2

Studies performed during normal lactation
Chapter 4

Respiratory burst activity of blood and milk neutrophils in dairy cows during different stages of lactation

This chapter is based on:

J. Mehrzad, H. Dosogne, E. Meyer, R. Heyneman and C. Burvenich

Abstract

The non-stimulated and phorbol 12–myristate 13–acetate (PMA) stimulated luminol-augmented cellular chemiluminescence (CL) response and viability of milk and blood polymorphonuclear leukocytes (PMN) were determined in lactating dairy cows during different stages of lactation. In the first study, 10 healthy cows in early, 10 in mid and 10 in late lactation were compared. In a second study, the same measurements as in the first study were evaluated longitudinally in 12 cows during 1 month following parturition. The CL activity and myeloperoxidase (MPO) content of milk PMN and macrophages (M) were also compared. Milk M did not possess MPO activity and were devoid of any luminol-enhanced CL. The CL activity of milk and blood PMN was significantly lower in early lactation than in mid- and late lactation (P < 0.001). Whereas little changes were observed in viability of blood PMN, the viability of milk PMN was lower in early lactation than in mid- and late lactation (P < 0.001). The % of PMN in isolated milk cells was also lower during early lactation than during mid-and late lactation (P < 0.001). The CL activity in response to PMA during early, mid-and late lactation increased 13, 59 and 42 – fold in blood PMN and 1.7, 2.6 and 2.4 – fold in milk PMN, respectively, in comparison with non-stimulated PMN. The CL activity, both in milk and blood PMN, the milk PMN viability and the % of milk PMN were lowest between 3 and 11 days post partum. These observed changes immediately after calving could contribute to a higher susceptibility to mastitis in that period.
1. Introduction

The outcome of bacterial infection is greatly dependent on the function of blood polymorphonuclear leukocytes (PMN). Inflammation of the mammary gland is characterized by a massive recruitment of circulating PMN into the gland, resulting in a dramatical increase of the number of milk cells (somatic cell count, SCC). An optimal function of blood PMN is essential for the first line defense mechanism of mammary gland. Impairment of blood PMN function at the onset of lactation has been associated with an increased susceptibility to bacterial infections (Burvenich et al., 1994). In contrast, the chemiluminescence (CL) of milk and blood PMN under physiological condition has been poorly investigated. The effectiveness of milk PMN to produce CL, which enables them to kill invading pathogens, is poorly understood. In addition, whereas the impaired respiratory burst activity of blood PMN in early lactating cows has been amply demonstrated (Kehrli et al., 1989; Detilleux et al., 1995; Dosogne et al., 1999; Hoeben et al., 2000), the kinetics of CL has not yet been characterized. The viability of blood PMN is generally accepted to be around 100 percent, but not much is known about milk PMN viability. Therefore, the viability of milk PMN was determined.

Apparently, milk PMN could be considered as rather ineffective cells, because of their short half-life time (± 8h), the exhaustion of their intracellular glycogen reserve (Naidu and Newbould, 1973), the induced apoptosis (Van Oostveldt et al., 1999) and their decreased respiratory burst activity (Smits et al., 1999). These effects are caused by diapedesis and by the inhibitory effects of protein and fat ingestion on intracellular killing (Russell et al., 1975; Paape et al., 1977). Nonetheless, although the function of milk PMN is decreased in comparison with blood PMN, several investigations have attributed a significant CL activity to milk PMN (Weber et al., 1983; Piccinini et al., 1999). The milk PMN function is modulated by the physiological conditions of the animal, in particular, the stage of lactation. For example, the CL activity of mammary gland PMN is lower in lactating cows than in heifers (Dulin et al., 1988).

Luminol-dependent CL is directly related to the presence of the granular enzyme myeloperoxidase (MPO; Rosen and Klebanoff, 1976; Dahlgren and Stendahl, 1983). At the start of this study, it was not clear whether milk macrophages (M) possess MPO activity. Due to the existence of a variety of cells: PMN, M, lymphocytes (L) and a small number of epithelial cells in nonmastitic milk, the cellular source of CL needed to be identified. Indeed, the contribution of the M to the CL was not known, and their presence in the isolated cells from milk samples could therefore interfere with the assessment of PMN function. To overcome this problem, a simple and rapid method was developed for the accurate assessment of the respiratory burst activity of milk PMN.

In order to obtain a better insight into the effects of the physiology of lactation on non-specific defense mechanisms within the mammary gland, the respiratory burst activity of milk and blood PMN and their viability during different periods of lactation was investigated.

2. Materials and Methods

Animals and experimental procedures

In total sixty Holstein-Friesian cows from the Ghent University dairy farm (Biocentrum Agri-Vet Melle, Belgium) were selected: cows in their first to third lactation, clinically healthy and free from mastitis. To confirm further that cows had
no mastitis pathogens, twice prior to experiment and once weekly throughout the experiment. 10 ml and 50 ml quarter-foremilk samples were collected, simultaneously, for bacteriological examination and determination of somatic cell count (SCC), respectively. Only cows with a quarter SCC < 2 × 10^5 cells/ml and milk samples that cultured negative for major mastitis pathogens were accepted for the study. In the first study, three different stages of lactation were investigated: early lactation cows (3 to 20 d after calving, n = 10), mid lactation cows (150 to 210 d after calving, n = 10), and late lactation cows (220 to 300 d after calving, n = 10). In the second study, 12 cows were selected for the CL assay in three different periods: from 3 to 11 d, 12 to 20 d, and 21 to 30 d after calving. The mean milk production per cow was 22.6 ± 1.9 l/d. For comparison of CL of milk PMN and M, 18 mid lactation cows were investigated. Mixed cisternal quarter milk samples (2 l; twice weekly for three weeks in the first study and six times in each period of the second study) were aseptically collected, after cleaning and disinfection of the teats, using a sterile teat cannula according to Vangroenweghe et al. (2000) and stored on ice for cell isolation and SCC determination. No additional stimulation of the mammary gland was used prior to milk collection. Simultaneously after milk sampling, blood samples (80 ml) were aseptically collected from the external jugular vein into evacuated tubes (Laboratoire EGA, Nogent-le Roi, France) containing 125 I.U. heparin for further process.

### Preparation of PMN from blood and milk

All materials and reagents used for the isolation of blood and milk PMN were sterile. Isolation of PMN from peripheral blood was performed using hypotonic lysis of erythrocytes according to Carlson and Kaneko (1973). After counting the cells using an electronically particle counter and determination of the viability and % of PMN as described below, the suspension was adjusted to a concentration of 5.10^6 cells/ml in Dulbecco's PBS (DPBS) (Gibco BRL, Life Technologies Inc., MD, USA) supplemented with gelatin (0.5 mg/ml, Merck, Darmstadt, Germany). Initial volumes of 2 l milk were processed using a high-capacity centrifuge (RC – 3BP, Sorvall, Newtown, CT, USA) after 60 % v/v dilution with cold phosphate buffered saline (PBS; 0.01 M phosphate buffer (KH2PO4-Na2HPO4) – 0.15 M NaCl, pH = 7.2). Fat was carefully removed after the first centrifugation (600 × g, 15 min, 4°C) and the pellet was washed twice at 300 × g for 10 min and 200 × g for 15 min at 4°C, in cold PBS, the cells were finally resuspended in DPBS supplemented with gelatin 0.5 mg/ml. After counting the isolated milk cells using an electronically particle counter, determination of viability and % of PMN as described below, the cells were resuspended to a concentration of 5.10^6 cells/ml in DPBS solution supplemented with gelatin 0.5 mg/ml, and finally assessed for respiratory burst activity. Milk cell recovery rate was calculated using the following formula, where RR = recovery rate, Ni = number of cells in isolated cell suspension, Vi = volume of isolated cell suspension (ml), SCC = somatic cell count in whole milk and Vm = volume of milk for isolation (ml).

\[
RR = \frac{N_i \cdot V_i}{SCC \cdot V_m}
\]

### Cell counting in blood and milk

The total number of circulating leukocytes and isolated cells from blood and milk were determined using an electronically particle counter (Coulter counter Z2, Coulter Electronics Ltd., Luton, UK). To do this, 25 µl of either whole blood or isolated cell suspensions was diluted in 20 ml of isotonic counting solution (Isoton® II, Counter
Respiratory burst activity of PMN during different stages of lactation

Electronics, D-47705 Krefeld, Germany). And 200 µl of lysis solution (containing (g/l) KCN 3.3, sodium nitroprusside 1.1, quaternary ammonium salt 55; Zap-Oglobin®; Coulter Electronics) was added to the suspension of whole blood. One hundred µl of the lysis solution was added to the suspension of isolated blood cells, whereas no lysis solution was used for the isolated milk cells. Determination of number of cells in the isolated counting solutions was applied by setting the instrument at 0.707 mA (milliamperere) with the dual threshold model. The lower size threshold (TL) for whole blood samples was used at 3.86 µm, whereas the TL for isolated cells from blood and milk was applied at 4.98 µm. The number of cells with an upper size than the TL was applied for quantification of cells in the sample. The counting procedure was performed in triplicate for each sample and the mean was calculated.

To quantify the yield of milk cell isolation, the SCC in whole milk was determined with a fluoro-opto electronic method (Fossomatic 400 cell counter, Foss Electric, Hillerød, Denmark).

Percoll isolation of milk PMN and M.

A Percoll isolation procedure was used in order to fractionate PMN from M. Briefly, 3 ml of 2 different Percoll (Pharmacia Biotech AB, Uppsala, Sweden) solutions with densities of 1.092 and 1.071g / l were layered in a 10 ml test tube. This gradient was carefully overlaid with 3 ml of a milk cell suspension and centrifuged at 400 × g for 25 min at 4°C. Upper, middle and bottom layers were obtained. Fractions were collected individually and washed (200 × g for 10 min at 4°C), counted and differentiated. The upper and bottom layers were used for further determination of purity and CL assay.

Viability of milk and blood PMN

The viability of isolated milk and blood PMN was determined by means of flow cytometrically (FACSScan, Becton Dickinson Immunocytometry Systems, San José, CA, USA). After addition of 10 µl propidium iodide (PI) (50 µg/ml PBS; Sigma Chemicals, St. Louis, Mo, USA) to 490 µl of diluted-isolated cells (1.10⁶ cells / ml DPBS, final concentration), the red fluorescence of PMN selected in the Forward Scatter (FS)-Side Scatter (SS) dot plot was determined. The correct gating of PMN in the FS-SS dot plot was based on two criteria: 1) flow cytometric analysis of percoll-purified PMN (confirmed by light microscopy); a linear positive correlation was found between % of PMN diagnosed by light microscopy and the PMN gated in the FS-SS dot plot (R = 0.98; P < 0.001; n = 48) 2) labeling of PMN with a specific monoclonal antibody (mAb) against bovine granulocytes (cell line CH 138A, VMRD, Inc., Pulman, WA, USA) and a secondary fluorescein isothiocyanate (FITC)-labeled antibody (Serotec Ltd., Oxford, England) (Davis et al., 1987). Red fluorescence of PI-incubated cells was registered at 650 nm using the 488 nm excitation wavelength. In the red fluorescence histogram, a gate (M1) was set to discriminate between live and dead cells with the lowest limit higher than autofluorescence. All cells within M1 gate were considered as dead. The threshold value for discrimination between live and dead cells was determined in isolated cells without PI addition, so that in samples without PI, 100 % cells were below the threshold value. The % of cells with a lower value than the threshold was used for calculation of the % of viability. The viability and % of PMN were measured both in the first and in the second studies. The coefficient of variation (C.V) for the viability of PMN in isolated cells averaged 3.7 ± 0.5 % for milk and 1.5 ± 0.4 % for blood.
Identification of blood and milk cells
Plasma was added to the isolated cells in a 1:1 ratio to prevent lysis during preparation of the smears. Differential cell counts on the isolates were performed on eosin-Giemsa-stained smears (Hemacolor® Merk Diagnostics, Darmstadt, Germany) using light microscopy. Differential leukocyte counts in blood were performed with light microscopy on whole blood smears stained with eosin-Giemsa. Identification of the cells in blood was based on morphological characteristics as described by Hayhoe and Flemans (1969). Cells differentiation in milk was based on morphological characteristics as described by McDonald and Anderson (1981). PMN was characterized by their multi-lobed or sometimes picnotic dark-bluish stained nucleus. M morphologically had a large size, a vacuolated nucleus and contained whitish fat globules in their cytoplasm. And L was either large with a low nucleus to cytoplasm ratio and high blue stained nucleus or small with a high nucleus to cytoplasm ratio and a dark bluish stained nucleus. Epithelial cells were identified as large, polygonal and uniform stained-light bluish cells. PMN (mature and immature), monocytes / M, L and epithelial cells (only in milk) were identified on 200 cells per slide. In blood cell identification procedure there was a positive correlation between the % of immature neutrophils in the cells from whole blood and isolated blood cells (R = 0.96; P < 0.001; n = 22; all were early lactation cows), indicating that isolated blood PMN are representative of whole blood PMN, thereby simplifying the interpretation of CL activity of isolated PMN.

Respiratory burst activity assay in milk and blood PMN
Luminol-enhanced PMA (phorbol 12-myristate 13-acetate)-stimulated cellular CL was used to measure the respiratory burst activity of cells isolated from blood and milk. Immediately after addition of 20 µl PMA (at final concentration of 100 ng / ml) and 60 µl luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione at final concentration of 0.3 mM, Sigma) to 2.10^6 cells / ml, the CL was measured in duplicate during 30 min at 37 °C with a microtiterplate luminometer (type LB96P; EG&G Berthold, D-75312 Bad Wildbad, Germany) using a total volume of 200 µl per well. Stock solutions of 200 µg / ml of PMA and 100 mM luminol were prepared in dimethyl sulphoxide (DMSO; Sigma) and always stored at -20 °C. The area under the curve (AUC) was calculated over a period of 30 min. The CL response was corrected for the actual number of viable PMN in each sample. Since we demonstrated that the contribution of milk M to CL is negligible (see results), the CL response was expressed per 10^3 viable PMN. The following formula was used to perform the corrections, where Cl = mean RLU / s, 4.10^5 = total number of cells per well, % PMN = total percentage of PMN in isolated cells, % V = percentage of viable PMN:

\[
\text{CL}_{\text{PMN}} \times 10^3 = \frac{\text{Cl}_{\text{isolated cells}} \times 10^6}{4.10^5 \times \% \text{PMN} \times \% V}
\]

The CL of blood PMN was calculated with the same formula as for milk PMN applying the corrections described by Heyneman et al. (1990) and Hoeben et al. (2000) for interference of eosinphil.

In addition, PMA stimulated CL was compared with non-stimulated CL both in blood and milk PMN. The profiles of the CL-curves of milk and blood PMN was also
performed in cows at (7 ± 3 d, n = 6), (160 ± 12 d, n = 6) and (265 ± 15 d, n = 6) after calving.
The respiratory burst activity was assessed both in the first and in the second studies.
The overall C.V of the CL assay was averaged 8.4 ± 2.1 % for milk PMN CL and 5.2 ± 1% for blood PMN CL.

**Contribution of PMN and M to the CL of milk cells**
The CL activity of pure PMN and M was calculated using the lower (PMN-enriched) and upper (M-enriched) fractions of isolated cells after Percoll density gradient separation. The following algebraic equations were used, where CL = mean RLU / s, CL\textsubscript{PMN} = CL activity of PMN, CL\textsubscript{M} = CL activity of M, % PMN = percentage of PMN in the isolated fractions, and % M = percentage of M in the isolated fractions:

\[
\begin{align*}
\text{CL}\textsubscript{upper} &= \text{CL}\textsubscript{PMN} \cdot \% \text{PMN} + \text{CL}\textsubscript{M} \cdot \% \text{M} \\
\text{CL}\textsubscript{bottom} &= \text{CL}\textsubscript{PMN} \cdot \% \text{PMN} + \text{CL}\textsubscript{M} \cdot \% \text{M}
\end{align*}
\]

CL\textsubscript{upper}, CL\textsubscript{bottom}, % PMN and % M are known variables, whereas CL\textsubscript{PMN} and CL\textsubscript{M} are unknown variables which can be calculated from the above algebraic equations.

**MPO staining technique**
The MPO staining technique was performed on smears of the isolated milk PMN and M fractions following Percoll separation. It consisted of benzidin dihydrochloride (BDHC) staining according to Kaplow et al. (1965) with some modification. Briefly, 20 mg BDHC / ml of 60 % v/v ethanolic solution and 0.5 mM of hydrogen peroxide at pH 6.2 dissolved and mixed for a final volume of 200 ml. Subsequently, 5 ml of fresh staining solution was added on the prepared smears, incubated for 10 min at 37°C, washed with tape water, air dried and finally counterstained with eosin-Giemsa Hemacolor® for microscopic examination.

**Statistical analyses**
Mean, SD and SEM were calculated by Statistix® Software, V. 4.1 (Analytical Software, Tallahassee, FL 32317, USA). In the first study, cows in each group were different but results within a group were normally distributed. In this study, analysis of variance (ANOVA) was used and means were performed by the least significant differences (LSD) test. In the second study cows within different period of early lactation were the same and were not normally distributed. Results between groups in early lactation were therefore compared using the Friedman test. Statistical analysis of the results was performed with the general model \( Y = \mu + S + C / S + e \). In this model, \( Y \) is the dependent variable, \( \mu \) the mean CL, \( S \) the stage of lactation and / or the period of early lactation, \( C \) the cow and \( e \) the experimental error term. Stage of lactation in the first study and period of early lactation in the second study were fixed factors and cows were randomized factor. Significant differences were determined at \( P < 0.05, 0.01 \) and 0.001.

Correlations between blood and milk PMN CL, CL and viability of milk PMN, immature neutrophils in whole blood and in isolated blood cells and percoll-purified PMN diagnosed by light microscopy and the PMN gated in the FS-SS dot plot were assessed using linear regression.
3. Results

Respiratory burst activity of milk and blood PMN
In the first study, the PMA–stimulated CL of PMN isolated from both milk and blood was lower in early lactation than in mid- and late lactation (P < 0.001). During mid- and late lactation it showed the same level in both matrices (Figure 1A). Moreover, non-stimulated CL of PMN was lower during early lactation than during mid- and late lactation but only in milk (P < 0.001). During mid- and late lactation it showed the same level in both matrices (Figure 1B). The ratio of PMA-stimulated to non-stimulated CL during early, mid- and late lactation was 1.8, 2.6 and 2.4 for milk PMN, whereas for blood PMN this ratio was 13, 60 and 42 during early, mid- and late lactation, respectively. In the second study, the PMA–stimulated CL of milk and blood PMN was lower during days 3 to 11, than during days 12 to 20 and 21 to 30 of after calving (P < 0.001, Figure 2A). In addition, non-stimulated CL of blood PMN was lower during days 3 to 11, than during days 12 to 20 and 21 to 30 of after calving (P < 0.5, Figure 2B). The ratio between PMA-stimulated to non-stimulated CL during days 3 to 11, 12 to 20 and 21 to 30 of lactation was 1.5, 2.0 and 2.3 for milk PMN, whereas for blood this ratio was 5.0, 14 and 16, respectively. The non-stimulated CL in blood PMN was similar in both studies (Figure 2A and 2B). A positive correlation was found between the PMA-stimulated CL of blood and milk PMN (R = 0.85; P < 0.001; n = 42).

Figure 3 A/B shows the CL profile of PMA-stimulated-milk and blood PMN during different stage of lactation. The CL profile typically showed biphasic pattern in stimulated blood PMN consisting of a low peak after approximately 3 minutes and reaching a maximal level after 12 minutes in mid-and late lactation. However, blood PMN during the early post partum period typically showed only the first lower peak of CL profile production. The intensity of CL of milk cells was markedly lower than in blood. Moreover, milk cells never demonstrated the biphasic pattern of blood cells and only revealed the early lower peak of CL during all stages of lactation.

Viability of milk and blood PMN
Figure 4 shows a typical result of the flow cytometric analysis of the milk PMN viability. PMN were selected in the FS-SS dot plot (Figure 4A). The red fluorescence of the PMN is used for the quantification of the % of viable PMN (Figure 4 B). The mAb against bovine granulocytes was used to confirm that all the milk cells selected in the FS-SS dot plot were PMN (Figure 4 C). In the first study, the viability of milk PMN was significantly lower in early lactation compared to mid- and late lactation cows (P < 0.001), whereas it was similar during mid- and late lactation (Table 1). In the second study, it was lower during days 3 to 11 than during days 12 to 20 and 21 to 30 of after calving (P < 0.001, Table 2). A positive correlation was found between total CL response of isolated milk cells and PMN viability (R = 0.91; P < 0.001; n = 42). The viability of blood PMN was similar both in the first and in the second studies.

Number and identification of milk and blood cells
The milk cell recovery rate was not dependent on the stage of lactation (Tables 1 and 2). The average % of PMN in isolated cells from milk was lower in early lactation than in mid- and late lactation. In addition, % PMN was lower during days 3 to 11 than during days 12 to 20 and-days 21 to 30 of early lactation (Tables 1 and 2). In the
first study, the total number of blood leukocyte and mature PMN count were lower during early lactation than during mid- and late lactation (P < 0.05; P< 0.01, respectively). In the second study, both numbers were also lower during days 3 to 11, than during days 12 to 20 and 21 to 30 of lactation (P<0.01; P< 0.001, respectively). The number of immature blood neutrophils, i.e band cells and (meta)myelocytes, was higher during early lactation than during mid and late lactation (P < 0.001). The number of immature blood neutrophils was also higher during days 3 to 11, than during days 12 to 20 and 21 to 30 of after calving (P < 0.001, Tables 1 and 2).

Chemiluminescence of milk PMN and M
The upper layer of the Percoll density gradient contained mostly M (70 ± 2%). In the bottom layer, the PMN were the predominant cell type with on average 92 ± 2% PMN and 6 ± 1% M. Quantification of the CL of both fractions and calculation by using above mentioned algebraic equations revealed that the CL activity of M was only 4 % of that of the PMN (Table 3).

MPO activity of milk cells
No MPO activity was observed in the granules of milk M, whereas in milk PMN the presence of dark blue stained granules clearly demonstrated the presence of MPO (Figure 5).

4. Discussion
In the present study the respiratory burst activity of both milk and blood PMN from healthy dairy cows has been evaluated during different stages of lactation. This is an original approach compared to other studies in which the mammary gland has been stimulated with lipopolysaccharide, oyster glycogen or an intramammary device in order to increase the number of PMN in milk (Weber et al., 1983; Saad, 1987; Dulin et al., 1988; Shuster et al., 1996), or with studies investigating the immune function of milk cells in mastitic cows (Piccinini et al., 1999). The present study investigated milk and blood PMN function under physiological conditions of the mammary gland, with a low SCC (less than 2.10^5 cells / ml). While the isolation procedure for blood yielded more than 95 % of pure and viable PMN the non-mastitic milk samples yielded an acceptable, but lower, and consisted of variable amount of PMN.

To obtain a sufficient amount of viable PMN from the low SCC milk samples, the isolation technique for milk was first optimized. Optimized conditions were also used for sample preservation. The careful manipulation of the milk samples yielded highly viable and sufficient PMN upon isolation. Phagocytosis of milk fat globules and casein micelles by milk PMN causes a decrease of PMN intracellular killing, phagocytosis and CL activity (Russell and Reiter, 1975; Jain and Lasmanis, 1978; Dulin et al., 1988). It may also cause a decrease in PMN viability. This phagocytosis represents one of the most important obstacles for milk PMN function assessment, and was stabilized by decreasing the temperature during the whole procedure of sampling and isolation.

Another factor that hampers the study of milk cells is the variability in cellular morphology and subsequently, the difficulty of cell differentiation which is necessary for the calculation of CL. Three major types of leukocytes were present in isolated milk cells: PMN, M and L. There was also a small % of epithelial cells. Epithelial cells and L do not influence the respiratory burst activity assay (Hallén Sandgren et
al., 1991), but PMN contributes to the CL activity of milk cells. At the start of this study, it was unclear whether M isolated from bovine milk had a significant CL activity. We have demonstrated that M in milk produce no luminol-dependent CL so that their interference with this assay is negligible. The explanation for this lack of CL activity is most likely that milk M have no MPO activity. Luminol has been used for quantification of the PMN respiratory burst during the CL assay based on measurement of the \( \text{H}_2\text{O}_2 \) production (Lind et al., 1983; Faulkner and Fridovich, 1993). An active MPO-\( \text{H}_2\text{O}_2 \) system is required for luminol-dependent CL (Rosen and Klebanoff, 1976; Dahlgren and Stendahl, 1983).

The knowledge that milk M produce no luminol-dependent CL, which is in accordance with observations by Hallén Sandgren et al. (1991), was of critical importance for the choice of the experimental set-up of milk PMN and to obtain unequivocal results. Indeed, the respiratory burst activity from milk cells measured in our assay can be solely attributed to the milk PMN, without interference of other milk cells like M. This knowledge allowed us to quantify the respiratory burst activity of PMN obtained from non-mastitis quarters with a low SCC and variable cellular composition.

The respiratory burst activity of PMN was impaired during early lactation both in blood and in milk in comparison with mid- and late lactation. Our results also indicate that the impairment of milk and blood PMN respiratory burst activity in high yielding dairy cows during early lactation is confined to the period between parturition and day 10 of lactation. In the study of Shuster et al. (1996) the cows within 10 days of lactation, by challenging intramammary of \textit{E. coli}, showed higher bacterial growth in the mammary gland and became more severely diseased compared to those during mid lactation. The ratio of milk to blood PMN CL was also lower in early (0.469) than in mid- and late lactation (0.782 and 0.754, respectively). Compared to blood PMN, the decreased CL activity of milk PMN may be explained by their ingestion of fat globules and casein micelles (Russell and Reiter, 1975; Paape and Guidry, 1977) and / or by their diapedesis through the blood / milk barrier (Smits et al., 1999). These processes could explain the decreased hydrogen peroxide production (Salgar et al., 1991) as well as the decreased PMA-stimulated CL (Weber et al. 1983). Our results are in agreement with the study of Dulin et al. (1988) observed the lower CL activity of milk PMN compared with blood PMN. The current study indicates that the physiological status of the dairy cow and in particular the stage of lactation can also be a considerable factor of influence on the CL activity of milk PMN. This could be related with the inevitable negative energy balance resulting in high concentration of ketone bodies, corticosteroids and parturition-and lactation-associated hormonal alterations (Hoeben et al., 1997; Moreira da Silva et al., 1998; Hoeben et al., 1999; 2000).

The observed decrease in blood PMN CL during early lactation is in agreement with the previously described decrease of respiratory burst activity of blood PMN (Kehrli et al., 1989; Heyneman et al., 1990; Shuster et al., 1996; Moreira da Silva et al., 1998; Dosogne et al., 1999; Hoeben et al., 2000). It has been demonstrated that other blood PMN functions such as chemotaxis (Kremer et al., 1993) and diapedesis (Vandeputte-Van Messom et al., 1993; Shuster et al., 1996) were also impaired during early lactation. These functions are acquired in a late stage of PMN maturation. The decreased respiratory burst activity in blood PMN could therefore be, in part, due to the presence of immature neutrophils. However, no immature neutrophils could be demonstrated in milk. This observation is in accordance with a previous study
Respiratory burst activity of PMN during different stages of lactation

(Moreira da Silva et al., 1998), in which the decreased respiratory burst activity of blood PMN could not solely be attributed to the presence of immature neutrophils. Therefore, the results of this study indicate that the respiratory burst activity of mature blood and milk PMN is impaired during early lactation. Because of the involvement of both superoxide anion and MPO in luminol dependent CL (Rosen and Klebabanoff, 1976) and because of the dependence on the MPO-H₂O₂ system (DeChatelet et al. 1982), it is likely that the MPO-catalysed bactericidal activity of milk and blood PMN, is impaired in early lactation.

A significant positive correlation was found between the CL response of milk and blood PMN. Apparently, the decreased blood PMN CL is maintained after transmigration through the blood/milk barrier. Hypothetically, the reduced CL of milk PMN might originate in the bone marrow. Moreover, hormonal and metabolic changes such as glucocorticoids, ketone bodies and pregnancy associated glycoproteins, which are characteristic for the early lactation period, impair PMN respiratory burst activity (Hoeben et al., 1997; Moreira da Silva et al., 1998; Hoeben et al., 2000). These hormones and metabolites also inhibit the proliferation of bone marrow cells in vitro (Hoeben et al., 1999).

The discrepancy between the high non-stimulated CL of milk PMN compared to blood PMN, indicates that milk PMN are already activated. This activation could be caused by known PMN activators such as β-lactoglobulin (Mehrzad et al., 2000a). The concentration of this PMN activating substance in milk is higher during mid and late lactation than during early lactation (Caffin et al., 1984). Enzymes activated by PMA, such as protein kinase C and NADPH-oxidase (Karlsson et al., 2000), could be less activated during the early post partum period. Further analysis is needed to identify PMN activators in milk during physiological conditions.

Interestingly, the profiles of cellular CL showed a difference between the milk and blood PMN. However, this difference was less pronounced during the early post partum period than during mid- and late lactation. Indeed, while the intensity of CL is lower in milk than in blood PMN, the plateau and shape of the milk-and blood CL curves were similar. Thus, the impairment of milk PMN CL during the early post partum seems to be directly related to that of the blood PMN. We demonstrated that the stimulated CL in blood PMN is maintained for at least 15 minutes during mid- and late lactation, whereas it is only maintained for less than 4 minutes during the early post partum period. Ineffectiveness of the oxygen-dependent intracellular bactericidal mechanism of blood PMN during the early postpartum period may be related to a shorter time of CL response towards pathogens. As the luminol-dependent system requires hydrogen peroxide (Lind et al., 1983; Faulkner and Fridovich, 1993) it is likely that the intracellular hydrogen peroxide production is higher in blood than in milk PMN. The decreased intracellular hydrogen peroxide production can be the cause of the significantly decreased of CL activity during the early post partum period both in blood and in milk. In addition, the disappearance of the second peak in the profile of milk PMN CL as well as a positive correlation between CL and bactericidal activity of milk PMN (Mehrzad et al., 2000b), we suggest oxygen-dependent intracellular killing of milk PMN toward pathogens is diminished, compared to blood PMN. It is unlikely that in the current study other milk cells contributed in a major part to this CL shape because even in the Percoll-separated PMN-enriched fraction (> 92 %) no second peak was observed either. According to Briheim et al. (1984), both intra- and extracellular events contribute to the luminol-dependent CL of human PMN. It could be proposed that the first peak is the result of the initial extracellular
reactions and the second peak is a result of subsequent intracellular reactions of the MPO-H2O2 system. The absence of a second peak in milk PMN during all stages of lactation and in blood PMN during the early post partum period needs to be further investigated.

In analogy with the respiratory burst activity, the viability of isolated milk PMN was substantially lower during early lactation (34 ± 5 %) than during mid- and late lactation (80 ± 2 % and 76 ± 3 %, respectively). This observation contrasts with the viability of blood PMN, which was consistently very high (near 100 %). The decrease in viability was also most pronounced within the first 10 days postpartum. In a recent study by Piccinini et al. (1999), the viability of milk PMN both in uninfected and in *Staphylococcus aureus* infected quarters was measured. The authors state a lower viability of milk PMN from uninfected quarters of an average 50 ± 19 % compared to 60 ± 20 % in pathological milk samples. Although no differentiation between early, mid- and late lactation is made, the viability values of PMN from uninfected quarters also range from about 30 to 70 % in the study of Piccinini et al. (1999). The physiological explanation for increased survival of milk PMN during mid- and late lactation and/or their impairment during early post partum period remains to be clarified. In addition, in our study the number of isolated PMN in milk was lower during early lactation than during mid and late lactation. This observation was also reported by Manlongat et al. (1998) in the goat. The higher % of PMN in milk during mid- and late lactation possibly results from the enhanced migration of circulating PMN into the milk during that period. A decreased % of PMN in milk can be another factor influencing compromised condition of the mammary gland against invading pathogens.

In conclusion, this is the first report of an impaired milk PMN CL, accompanied by a decreased viability in early lactation, more specifically during the 1st 10 d of post partum. During early lactation, respiratory burst activity of blood and milk PMN and the number and viability of milk PMN were decreased. Together, these findings suggest an impairment of PMN bactericidal capacity during early lactation. This general impairment could play a role in the increased incidence of infectious diseases such as mastitis in high yielding dairy cows during the early post partum period.

This study was supported by the Belgian Ministry of Small Enterprises and Agriculture (Grant S/5871), by the Belgian Fund of Scientific Research (FWO/FNRS Grant 3G008699) and by the Ministry of Science, Research and Technology of Iran. The authors wish to thank L. De Bruyne, M-R De Smet and E. Vander Elstraeten for their excellent technical assistance.

**References**


Chapter 4


Shuster DE., Lee EK. and Kehrli ME. 1996. Bacterial growth, inflammatory cytokine production, and neutrophil recruitment during coliform mastitis in cows within ten
days after calving compared with cows at mid lactation. Am. J. Vet. Res. 57: 1569-1576.
Table 1. Parameters of milk and blood cells measured during the first study in early, mid and late lactation cows. Values are means ± SEM of 10 cows.

<table>
<thead>
<tr>
<th>Days after calving</th>
<th>3 - 20</th>
<th>150 – 210</th>
<th>220 - 300</th>
</tr>
</thead>
<tbody>
<tr>
<td>Somatic cell count (SCC; × 1000 / ml)</td>
<td>43.5 ± 19.9**</td>
<td>81.6 ± 18.2</td>
<td>90.3 ± 23.8</td>
</tr>
<tr>
<td>Milk cell recovery rate (%)</td>
<td>33 ± 4</td>
<td>31 ± 5</td>
<td>29 ± 3</td>
</tr>
<tr>
<td>Isolation of milk PMN (%)</td>
<td>46 ± 4***</td>
<td>69 ± 3</td>
<td>70 ± 2</td>
</tr>
<tr>
<td>Viability of blood PMN (%)</td>
<td>98 ± 1</td>
<td>98 ± 1</td>
<td>97 ± 0.5</td>
</tr>
<tr>
<td>Viability of milk PMN (%)</td>
<td>34 ± 5***</td>
<td>80 ± 2</td>
<td>76 ± 3</td>
</tr>
<tr>
<td>Total number of leukocytes /µl blood</td>
<td>8272 ± 482*</td>
<td>8690 ± 450</td>
<td>9815 ± 400</td>
</tr>
<tr>
<td>Total number of circulating mature PMN / µl blood</td>
<td>2232 ± 412**</td>
<td>3108 ± 292</td>
<td>3149 ± 277</td>
</tr>
<tr>
<td>Total number of circulating band neutrophils / µl blood</td>
<td>429 ± 77***</td>
<td>12 ± 8</td>
<td>0</td>
</tr>
<tr>
<td>Total number of circulating myelocytes and metamyelocytes / µl blood</td>
<td>824 ± 187***</td>
<td>10 ± 7</td>
<td>0</td>
</tr>
</tbody>
</table>

** = P < 0.01 *** = P < 0.001

Table 2. Parameters of milk and blood cells measured during the second study in three different periods of early lactation. Values are means ± SEM of 12 cows.

<table>
<thead>
<tr>
<th>Days after calving</th>
<th>3 - 11</th>
<th>12 - 20</th>
<th>21 - 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Somatic cell count (SCC; × 1000 / ml)</td>
<td>58.5 ± 10.5*</td>
<td>46.7 ± 13.1</td>
<td>44.5 ± 9.7</td>
</tr>
<tr>
<td>Milk cell recovery rate (%)</td>
<td>37 ± 5</td>
<td>34 ± 4</td>
<td>32 ± 4</td>
</tr>
<tr>
<td>Isolation of milk PMN (%)</td>
<td>26 ± 2***</td>
<td>39 ± 2</td>
<td>40 ± 3</td>
</tr>
<tr>
<td>Viability of blood PMN (%)</td>
<td>99 ± 0.2</td>
<td>98 ± 0.2</td>
<td>98 ± 0.3</td>
</tr>
<tr>
<td>Viability of milk PMN (%)</td>
<td>27 ± 2***</td>
<td>42 ± 2</td>
<td>49 ± 3</td>
</tr>
<tr>
<td>Total number of circulating leukocytes / µl blood</td>
<td>7547 ± 425**</td>
<td>9341 ± 392</td>
<td>9458 ± 471</td>
</tr>
<tr>
<td>Total number of circulating mature PMN / µl blood</td>
<td>1938 ± 15***</td>
<td>3746 ± 232</td>
<td>3776 ± 272</td>
</tr>
<tr>
<td>Total number of circulating band neutrophils / µl blood</td>
<td>601 ± 57***</td>
<td>341 ± 38</td>
<td>293 ± 41</td>
</tr>
<tr>
<td>Total number of circulating myelocytes and metamyelocytes / µl blood</td>
<td>860 ± 109***</td>
<td>462 ± 54</td>
<td>338 ± 83</td>
</tr>
</tbody>
</table>

** = P < 0.01 *** = P < 0.001
Table 3. Luminol-dependent chemiluminescence (CL) and cellular composition (PMN = polymorphonuclear neutrophils, M = macrophage and L = lymphocytes) of the recovered fractions after Percoll density gradient centrifugation. Values are means ± SEM of 18 cows.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>PMN (%)</th>
<th>M (%)</th>
<th>L (%)</th>
<th>CL (^a) (RLU/s ×10^5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMN-enriched</td>
<td>92 ± 1.3</td>
<td>6.2 ± 1.3</td>
<td>1.4 ± 0.6</td>
<td>8.46 ± 0.9</td>
</tr>
<tr>
<td>M-enriched</td>
<td>26 ± 1.2</td>
<td>70 ± 1.8</td>
<td>3.8 ± 0.8</td>
<td>0.36 ± 0.1</td>
</tr>
</tbody>
</table>

\(^a\) CL is expressed as mean relative light units / second (RLU/s).
Figure 1. Chemiluminescence of PMA stimulated (panel a) and non-stimulated (panel b) of PMN isolated from blood and milk during early, mid- and late lactation. Values are expressed as the area under the curve (AUC) of RLU / S of $10^3$ viable PMN during 30 minutes. Values are means ± SEM of 10 cows. Significance of the difference between different stages of lactation is indicated with asterisks (***: $P < 0.001$).
Figure 2. Chemiluminescence of PMA stimulated (upper panel) and non-stimulated (lower panel) of PMN isolated from blood and milk during the 3 different periods of early lactation. Values are expressed as the area under the curve (AUC) of RLU / S of $10^3$ viable PMN during 30 minutes. Values are means ± SEM of 12 cows. Significance of the difference between different periods of early postpartum is indicated with asterisks (*: P < 0.05 ***: P < 0.001).
Figure 3. Chemiluminescence shape of PMA-activated luminol enhanced of milk (a) and blood (b) isolated cells during different period of lactation. The top through the bottom curves depict the CL shape of the isolated cells at 265 ± 15 d, 160 ± 12 d and 7 ± 3 d after parturition respectively. The Y axes is the cumulative RLU / s in function of time, and the X axes is the entire measurement period of CL. Curves are means ± SEM of 6 cows.
Figure 4. Flow cytometric analysis of isolated bovine milk PMN gated in the FS-SS dot plot (A). B: green fluorescence of PMN labeled with a monoclonal antibody specific against bovine granulocytes and with a secondary FITC-labeled antibody. C: red fluorescence of propidium iodide-incubated PMN selectively gated in the FS-SS dot plot. Gate M1 is applied to determine the percentage of dead PMN (for the quantification of viability).
Figure 5. Light microscopic image of PMN (middle), a macrophage (A) and a lymphocyte (B) isolated from non-mastitic milk stained with benzidine dihydrochloride and counterstained with hematoxilin-eosin (× 1000).
Chapter 5

Blood and milk neutrophil chemiluminescence and viability in primiparous and pluriparous dairy cows during late pregnancy, around parturition and early lactation

This chapter is based on:

J. Mehrzad, L. Duchateau, S. Pyörälä and C. Burvenich

Abstract

Extensive studies have shown the polymorphonuclear leukocytes (PMN) dysfunction inextricably links to parturition. To investigate the effect of parity on PMN function, phorbol 12–myristate 13–acetate (PMA) stimulated luminol-amplified chemiluminescence (CL) and viability of blood and milk PMN were investigated in primiparous and pluriparous dairy cows during periparturient period. The CL kinetics of blood and milk PMN and haematological profiles were also assessed. Milk PMN CL was always lower than blood PMN CL. Blood and milk PMN CL and milk PMN viability were significantly higher in primiparous cows throughout the study. Blood PMN CL in pluriparous cows showed a sharper decrease. Both in pluriparous and in primiparous cows, minimal blood PMN CL appeared at periparturient day (ppd) 2. After ppd 7, blood PMN CL recovery rate was faster in primiparous cows. Milk PMN CL was minimal at ppd 2 in both groups. Whereas no changes were observed on blood PMN viability, the viability of milk PMN in primiparous cows was substantially higher than in pluriparous cows. Number of circulating eosinophils and immature neutrophils was substantially higher in primiparous cows throughout the study. The CL kinetics of blood PMN at ppd –2 and 2 and of milk PMN at ppd 2 exhibited different responses to PMA, with higher intensity and durability, peaking and subsiding more slowly in primiparous dairy cows. The pronounced reduction in PMN CL and viability in milk PMN of pluriparous cows may be involved in the underlying mechanisms that make these animals more susceptible to periparturient infectious diseases.
1. Introduction

Bovine blood and milk PMN have the potential to produce reactive oxygen species (ROS) to eventually kill engulfed bacteria (Weber et al., 1983; Dulin et al., 1988; Kehrli et al., 1989; Mehrzad et al, 2001a; b). It is widely accepted that PMN ROS production is an important defense mechanism against Gram-negative-and positive bacteria (see e.g. Burvenich et al., 1994). Quantification of ROS can be measured following PMN stimulation with soluble agents, e.g. PMA or with particles e.g. zymosan, bacteria, latex beads. The most widely used technique to quantify PMN ROS production is chemiluminescence (CL) assay (Allen et al., 1972; Weber et al., 1983; Piccinini et al., 1999). The different CL responsiveness of blood and milk PMN to PMA stimulation during physiological conditions could result from differences in protein kinase C, NADPH-oxidase and myeloperoxidase (MPO) activities (Webb et al., 1974; Babior, 1984). As these enzyme activities reflect intracellular and extracellular reactions, changes might offer some evidence about the cow’s susceptibility to early lactation-related infectious diseases.

The transition from pregnancy to lactation causes stress in dairy cows. This transition is accompanied by a gradual decrease in PMN CL (Moreira da Silva et al., 1998, Hoeben et al., 2000). The decrease in PMN MPO activity (an index of PMN CL) has also been reported in mastectomized cows during the same period (Kimura et al., 1999). Furthermore, the immunocompromising effect of parturition is boosted by lactogenesis (Shuster et al., 1996, Goff et al., 1997, Mehrzad et al., 2001a). The severity of coliform mastitis has been reported to be less pronounced in animals with higher pre-infection PMN ROS production (Heyneman et al., 1990; Vandeputte-Van Messom et al., 1993). Increased severity of dairy cows to E. coli mastitis has also been associated with increased parities (van Werven et al., 1997).

In this paper the effect of parity on the PMN CL suppression that occurs around parturition was studied. CL was studied both in blood and milk PMN. PMN viability was also included because it was recently found that it is also influenced by lactation cycle (Mehrzad et al., 2001a). The kinetics of blood and milk PMN CL was further studied in detail.

2. Materials and Methods

Animals and experimental procedures

In total twenty four Holstein-Friesian cows from the Ghent University dairy farm (Biocentrum Agri-Vet Melle, Belgium) were selected: cows in their first to fifth lactation, clinically healthy and showing no sign of typical periparturient diseases after calving. To confirm that cows had no mastitis pathogens, 10 ml and 50 ml quarter-foremilk samples were collected after parturition once weekly throughout the experiment and examined for bacteriological infection and somatic cell count (SCC), respectively. The animals were parasitologically negative, and there was no distinguishable allergic caused by ecto-and/or endoparasites. Only cows with a quarter SCC < 2 × 10^5 cells/ml and milk samples that cultured negative for major mastitis pathogens were considered as clinically healthy. Parities were combined as follows: primiparous cows (first gestation, 2.3±0.4 yr, n=12) and pluriparous cows (forth to fifth gestation, 5.7±0.6 yr, n=12). The mean milk production for primiparous and pluriparous cows was 17.2 ± 1.3 l/d and 23.7 ± 2.3 l/d, respectively. Before calving (from 32±3 days before parturition), 80 ml blood samples were aseptically collected.
twice weekly from the external jugular vein into evacuated tubes (BD-Vacutainer System, Plymouth; UK) containing 125 IU heparin for further processing. After calving (until 32±3 days after parturition), 1.5 L mixed cisternal quarter milk samples were aseptically collected in the morning twice weekly after cleaning and disinfection of the teats, using a sterile teat cannula (Vangroenweghe et al., 2001) and stored on ice for cell isolation and SCC determination. No additional stimulation of the mammary gland was used prior to milk collection. After milk sampling, blood samples were collected for further processing using the same procedure as before calving. To compare blood and milk parameters between the primiparous and pluriparous cows, the two groups were classified at three different periods: 1) 3 days before parturition until parturition, 2) from parturition until 7 days after parturition and 3) from more than 7 days until 5 weeks after parturition.

Blood and milk PMN preparation and enumeration
All materials and reagents used for the isolation of blood and milk PMN were sterile. Isolation of PMN from peripheral blood was performed using hypotonic lysis of erythrocytes (Carlson and Kaneko, 1973). The isolation procedure of PMN from blood yielded > 98% of granulocytes (PMN + eosinophils) with predominantly PMN (> 86%) and a viability of > 98% in both groups. After counting the cells using an electronic programable particle counter (Coulter counter Z2, Coulter Electronics Ltd., Luton., UK) and determining the viability and percentage of PMN, the cell suspension was adjusted to a concentration of 5.10^6 cells/ml in Dulbecco's phosphate buffered saline (DPBS; Gibco BRL, Life Technologies Inc., MD, Gaithersburg) supplemented with gelatin (0.5 mg/ml; Merck, Darmstadt, Germany). Initial volumes of 1.5 L milk were processed using a high-capacity centrifuge (RC – 3BP, Sorvall, Newtown, CT, USA) after 60% vol/vol dilution with cold phosphate buffered saline (PBS; 0.01 M phosphate buffer (KH_2PO_4-Na_2HPO_4) – 0.15 M NaCl, pH = 7.2). Isolation of PMN from milk was performed using three centrifugation steps as previously described (Mehrzad et al., 2001a), yielding > 60% PMN with different viability throughout the experiment. The isolation procedure “time” for milk PMN was similar to that of blood PMN (54±3 min). To quantify the yield of milk cell isolation, the SCC in whole milk was determined with a fluoro-opto electronic method (Fossomatic 400 cell counter, Foss Electric, Hillerød, Denmark). Milk cell recovery rate was calculated using RR = (N_i . V_i)/SCC . V_m, in which RR = recovery rate, N_i = concentration of cells in isolated cell suspension, V_i = volume of isolated cell suspension (ml), SCC = somatic cell count in whole milk and V_m = volume of milk for isolation (ml). The overall recovery rate throughout the study yielded 35% ± 3.6 and 33% ± 4.2 in primiparous and pluriparous cows, respectively. The total number of leukocytes and isolated blood and milk cells were determined using an electronic particle counter (Mehrzad et al., 2001a; b).

The total number of different circulating leukocytes was determined using smear preparation of blood sample (Mehrzad et al., 2001a; b). Similar whole blood staining procedure was performed to isolated blood and milk cells.

Viability and identification of blood and milk PMN
The viability of isolated PMN was determined in duplicate by means of flow cytometry (FACSScan, Becton Dickinson Immunocytometry Systems, San José, CA, USA), using propidium iodide exclusion (Mehrzad et al., 2001a).
Differential cell counts on the isolates were performed on eosin-Giemsa-stained smears, using light microscopy. Identification of the cells on isolates and whole blood was based on morphological characteristics as described by (Hayhoe and Flemans, 1969). Isolated milk cell differentiation was based on morphological characteristics (McDonald and Anderson, 1981), with some modification (Mehrzad et al., 2001a). Overall, macrophages had a large size, a vacuolated nucleus and contained whitish globules in their cytoplasm. Large Lymphocytes were with a regular and dark bluish-stained nuclei, however monocytes were with their less condense and irregular nuclei and always higher cytoplasm to nucleus ratio. Epithelial cells, though less than 3% (negligible) in milk “isolates”, were identified as large, polygonal and uniform stained-light bluish cells.

To quantify percentages of each cell type in the samples PMN (mature and immature), monocytes/macrophages, lymphocytes, eosinophils and epithelial cells (only in milk) were identified on 200 cells per slide and expressed as % of particular cells in respective samples.

**Chemiluminescence assay**
Luminol-enhanced PMA-stimulated cellular CL was used to quantify the ROS production of PMN isolated from blood and milk (Mehrzad et al., 2001a). The area under the curve (AUC) was calculated over a period of 30 min. The CL response was corrected for the actual number of viable PMN in each sample. As we previously demonstrated that the contribution of milk macrophages to luminol-dependent CL is negligible (Mehrzad et al., 2001a), the CL response was expressed per $10^3$ viable PMN. For milk PMN CL assay the formula, $\text{CL}_{\text{PMN}} = 10^3 \times \frac{\text{Cl}_{\text{isolated cells}}}{(4.10^5 \times \% \text{PMN} \times \% \text{V})}$, was used to perform the corrections, where $\text{Cl} = \text{mean RLU (relative light unit)/s}$, $4.10^5 = \text{total number of cells per well}$, $\% \text{PMN} = \text{total percentage of PMN in isolated cells}$, $\% \text{V} = \text{percentage of viable PMN}$. The CL of blood PMN was calculated with the same formula as for milk PMN applying the corrections described by Heyneman et al. (1990) and Hoeben et al. (2000) for interference of eosinophils.

In addition, the kinetics of ROS production of blood PMN was compared between primiparous and pluriparous cows at ppd -2 and 2; and for milk PMN this comparison was performed at ppd 2.

**Statistical analyses**
The difference between primiparous and pluriparous cows for blood PMN CL and viability was assessed and tested at three different periods: 1) 3 days before parturition until parturition, 2) from parturition until 7 days after parturition and 3) from more than 7 days until 5 weeks after parturition. The first two analyses were based on a mixed model with cow as random effect and the actual day of measurement and parity as fixed effects. The third analysis was based on a mixed model with cow as random effect and parity as fixed effect, but time was now introduced as a continuous fixed effect and further the interaction between time and parity was added.

The same analyses were done for milk PMN CL and viability, but obviously only for periods 2 and 3.

All other blood and milk parameters were analyzed in the same way, but are considered to be exploratory analyses. Therefore, the results are only summarized in terms of differences (with their 95% confidence interval) between primiparous and pluriparous cows for the relevant periods.
3. Results

**Chemiluminescence of blood and milk PMN**

The overall PMA-simulated CL of blood PMN was lower in pluriparous than in primiparous cows in the three periods studied (P<0.0001) (Figure 1a,b; Table 1). The relative magnitude of the blood PMN CL differences in pluriparous cows during the periods 1, 2 and 3 was 59%, 32% and 62% of those of primiparous cows, respectively. Blood PMN CL showed a sharp decrease during periparturient period both in the pluriparous and in primiparous cows. The decline in blood PMN CL was largest in the second study period, immediately after parturition, and went below AUC of 2000 for all pluriparous cows, whereas it stayed above this level for all primiparous cows (Figure 1a). In the third period, blood PMN CL recovery rate was significantly faster in primiparous cows than in pluriparous cows (Table 1).

Throughout the whole study period, milk PMN CL was always lower than that of blood PMN both in primiparous and pluriparous cows. Additionally, the milk PMN CL was consistently higher in primiparous than in pluriparous cows in both periods studied (p <0.01; Figure 1b). The relative magnitude of the milk PMN CL differences in pluriparous cows during the periods 2 and 3 was 62% and 78% of those of primiparous cows, respectively.

Figure 2 shows the kinetics of CL after PMA stimulation in blood and milk PMN of five primiparous cows and five pluriparous cows during the first and the second period. During these periods the lowest CL values were observed. Although blood PMN CL in primiparous cows at ppd –2 was higher than the CL in pluriparous cows, the RLU max reached faster in pluriparous cows. Similarly, at ppd 2 the CL peaked faster in pluriparous cows. Furthermore, the CL response by primiparous PMN decreased more slowly than the pluriparous PMN CL response. Beyond the slightly lower milk PMN CL intensity in pluriparous cows at ppd 2, the CL peak in milk PMN appeared faster in pluriparous cows than those of primiparous cows.

**Viability of blood and milk PMN**

No differences were observed for blood PMN viability of primiparous and pluriparous cows in the three study periods (Figure 3a), and blood PMN viability was always high. The viability of milk PMN was lower in pluriparous cows compared to primiparous cows in the two periods studied (P < 0.001; Figure 3b). In contrast, on ppd 2, 3 and 4 SCC in pluriparous cows was higher than in heifers (39000 cells/ml). From ppd 7 onward, no differences in SCC could be detected between both groups (Table 1).

**Enumeration of leukocytes and leukogram**

The largest difference in total white blood cell count and number of mature neutrophils between primiparous and pluriparous cows was observed in period 1, before parturition, with the difference equal to 3719/µl for white blood cells (WBC) and 2604/µl for mature neutrophils. In the two following study periods the difference was much smaller. The largest difference in band neutrophils in favor of the primiparous cows was observed immediately after parturition and a substantial difference was also noted in the first study period, immediately before parturition (Table 1). Finally, the number of metamyelocytes and myelocytes and eosinophils in primiparous cows was higher than in pluriparous cows in any of the three study periods.
4. Discussion

Marked suppression of blood PMN CL was observed around parturition and during early lactation in primiparous and pluriparous dairy cows. Although we did not study any cause-and-effect relationship between periparturition and PMN dysfunction, such an association with increased cow’s susceptibility to mastitis pathogens was nonetheless reported elsewhere (Kehrli et al., 1989; Kremer et al., 1993; Shuster et al., 1996). This relationship might contribute to the severity of coliform mastitis during early lactation that links to particularly “parity” (van Werven et al., 1997). Our results also indicate that the impairment of blood PMN CL in primiparous and pluriparous dairy cows during periparturition is confined to the period between periparturient week (PPW) –1 and 2. This is in accordance with previous reports (Moreira da Silva et al., 1998; Kimura et al., 1999, Hoeben et al., 2000), indicating that the transitional period from parturition to lactation is immunosuppressive. This, in part, lead to an overall impaired bactericidal capacity of blood PMN during early lactation reported in vivo (Shuster et al., 1996) and in vitro (Mehrzad et al., 2001a). While the role of ROS in PMN bactericidal activity is still the subject of debate (Reeves et al., 2002), it is generally agreed that PMN ROS is pivotal for bactericidal activity (Burvenich et al., 1994). Indeed, PMA-stimulated PMN CL was the central part of the current study. A previous study unequivocally revealed a positive correlation between PMA-stimulated PMN CL and their bactericidal activity as well as with latex-stimulated PMN CL (Mehrzad et al., 2001b). Substantial evidence also revealed that PMN phagocytosis and CL are interrelated, which conclusively gives PMN bactericidal activity (Allen et al., 1972; Grebner et al., 1977).

There is no doubt that when bacterial invasion occurs, especially with Gram-negatives, ROS production will facilitate pathogen elimination. When ROS is produced adequately, cell and tissue damage will be far less than when the pathogen is not eliminated. Uncontrolled generation of ROS is harmful for many cell systems e.g. T cell hyporesponsiveness and lymphocyte proliferation inhibition caused by ROS (Nonnecke and Harp, 1988; Cemerski et al., 2002). On the other hand, Nonnecke and Harp (1988) observed that blood and milk PMN by phagocytosis of *Staphylococcus aureus* inhibits lymphocyte cytotoxicity and enhances mononuclear cell viability. ROS also differently enhances natural killer cell and T cell activity (Suthanthiran et al., 1984; Cemerski et al., 2002), indicating that PMN ROS may not only damage to the cells and tissues but may also accelerate recovery of inflammation e.g. mastitis. It is still debatable that the modulation caused by PMN might be somewhat different in heifers and pluriparous cows around parturition. Other blood PMN functions such as chemotaxis (Kremer et al., 1993) and diapedesis (Hill et al., 1979; Vandeputte-Van Messom et al., 1993; Shuster et al., 1996) were compromised during early lactation. The suppression in PMN CL has been found to be associated with the sudden changes in concentrations of ketone bodies (Moreira da Silva et al., 1998; Suriyasathaporn et al., 1999) glucocorticosteroids (Guidry et al., 1976), and pregnancy and lactation-associated molecules (Dosogne et al., 1999; Hoeben et al., 2000). Moreover, PMN CL in milk was lower than in blood in both primiparous and pluriparous dairy cows. The lower milk PMN CL after stimulation with PMA was also seen in other studies (Weber et al., 1983; Dulin et al., 1988; Mehrzad et al., 2001a). This may be explained by their exhausted state through ingestion of fat globules and casein micelles (Russell and Reiter, 1975; Paape and
Guidry, 1977) and/or by the effect of diapedesis through the blood/milk barrier (Smits et al., 1999). Diapedesis also caused apoptosis (Van Oostveldt et al., 2002), which lowered milk PMN CL.

The current study indicates that parity of the dairy cow influences blood PMN CL. As far as PMN ROS production capacity is concerned, the well-known alteration of PMN function seems to be more depressed in pluriparous cows. Because of the involvement of both superoxide anion and MPO–H₂O₂ system in luminol dependent CL (Rosen and Klebanoff, 1976; DeChatelet et al., 1982), it is likely that the MPO-catalysed bactericidal activity of blood PMN is more active in primiparous dairy cows. Other blood PMNs (e.g., immature neutrophils) were observed in isolated blood cells with a higher frequency in primiparous cows. This could have further lowered blood PMN CL in primiparous cows as they produce less ROS than mature neutrophils (Glasser et al., 1987). But blood PMN CL was higher in primiparous cows around parturition and during early lactation, potentially revealing “even higher immature neutrophil CL” in primiparous cows than in pluriparous cows. This observation further supports our hypothesis that oxidation-reduction reactions in primiparous neutrophils could be more functional than those of pluriparous.

Our results also indicated that the milk PMN CL was minimal at PPW 1 both in primiparous and pluriparous cows. This is in agreement with previous results (Mehrzad et al., 2001a). What was unpredictable is the higher milk PMN CL in primiparous dairy cows during early lactation. Indeed, following milk PMN stimulation with PMA the decreased ROS production capacity resulted mainly from already ingestion of fat and casein in the milk compartment (Russell and Reiter, 1975; Paape and Guidry, 1977). This indicates that, in fact, milk PMN behave as pre-stimulated cells, rendering them less responsive toward PMA, possibly implying “exhausted state” for milk PMN CL. As the current result revealed, this potential exhausted state would be more peculiar for pluriparous cows, of which influencing factors remain to be clarified. According to Zeconci et al. 1994 and current observation, it can be concluded that the higher probability of developing clinical mastitis in pluriparous cows would result, at least in part, from lower milk PMN CL.

The kinetics of cellular CL of blood and milk PMN showed further disparities between the primiparous and pluriparous cows. In addition to a longer onset time for primiparous blood PMN CL at ppd -2 and 2, the PMA-stimulated CL at ppd -2 is cumulatively remained for at least 13 min in primiparous cows, whereas it is only maintained for less than 5 min in pluriparous cows. This shorter time of CL response towards stimuli potentially results in a less effectiveness of the oxygen-dependent intracellular bactericidal mechanism of blood PMN around parturition in pluriparous dairy cows. Furthermore, the PMA stimulated CL at ppd 2 is also, of course, less intense than those of ppd-2 and increased for at least 11 min in primiparous cows, while in pluriparous it was only peaked around 4 min and then subsided. This also demonstrates that there is less intracellular bactericidal efficiency in pluriparous dairy cows at ppd 2. Although several intracellular bactericidal mechanisms have been described, evidence exists that the production of ROS is one of the most important killing mechanisms, especially for Gram-negatives. Moreover, the shape of the kinetic events during PMA-induced luminol-dependent CL reveals some details on the location of the ROS that are produced intra-and-extracellularly (Rosen and Klebanoff, 1976; DeChatelet et al., 1982). The luminol dependent CL kinetics after 3-4 minutes are considered to be the result of intracellular events (DeChatelet et al., 1982;
Edwards et al., 1986; Faulkner et al., 1993). As the luminol-dependent CL requires hydrogen peroxide (Lind et al., 1983; Edwards et al., 1986; Faulkner and Fridovich, 1993) it is likely that the intracellular hydrogen peroxide production is higher in primiparous blood PMN than in pluriparous ones. Subsequently, impairment of intracellular reactions of MPO–H₂O₂ system is more pronounced in pluriparous dairy cows. The decreased intracellular events of ROS production could be the main cause of the significant decrease of CL activity immediately before and after parturition both in primiparous and pluriparous cows. The slightly higher intensity and peak in primiparous milk PMN CL suggests that the diminished oxygen-dependent intracellular killing of milk PMN toward pathogens is more pronounced in pluriparous cows. As previously reported (Mehrzad et al., 2001a), it is unlikely that in the current study other milk cells contributed significantly to this CL shape.

Analogous with CL, the milk PMN viability was substantially higher in primiparous than in pluriparous dairy cows during early lactation. This observation contrasts with the viability of blood PMN, which was consistently very high (~100 %). The minimal milk PMN viability was also most pronounced within the first week postpartum. This is in accordance with our previous study (Mehrzad et al., 2001a). In a recent study (Piccinini et al., 1999), though no differentiation between lactation number is made, the viability values of PMN from uninfected quarters also ranges from 30 to 70 %. Another comparative study (Van Oostveldt et al., 2001) revealed also lower viability of milk PMN during early lactation. The exact mechanism for higher survival of milk PMN in primiparous dairy cows and/or their more pronounced impairment in pluriparous cows remains unknown. There are, however, some possible explanations for this discrepancy. According to studies on human and bovine PMN (Mayer et al., 1989; Jankowski et al., 2002), PMN NADPH-oxidase activity (an index of PMA-induced PMN CL) and their viability are interrelated. PMA is a potent NADPH-oxidase and protein kinase-C agonist (Karlsson et al., 2000). As the NADPH-oxidase is a trigger of PMN respiratory burst and proton transportation into the PMN phagosomal and cytosolic space, the enzyme’s activity is regulated by MPO as well (Edwards et al., 1986). The lower milk PMN CL in pluriparous cows suggests NADPH-oxidase and MPO activity impairment. The contribution of neutrophil NADPH-oxidase activity is pivotal for their viability (Mayer et al., 1989; Jankowski et al., 2002). Furthermore, NADPH-oxidase activity contributes to phagosomal and cytosolic pH homeostasis (Reeves et al., 2002), maintaining PMN stoichiometry and electroneutrality (Takanaka et al., 1988). This phenomenon helps prevent “rapid” cytosolic acidification and necrosis, and thus might have had an effect on PMN survival in our study. This preliminary evidence supports the assumption of rapid cytosolic milk PMN acidification in pluriparous cows, which could result in a faster PMN necrosis. However, there could also be other physiological factors, such as recruitment of younger neutrophils in the milk compartment of primiparous cows, which might involve the delay of apoptosis and the increase of viability. The consistently higher milk PMN viability in primiparous cows suggests that primiparous cows might be a better source of milk PMN. Similarly, the higher quality of blood PMN would make primiparous cows as a better PMN donor for in vitro test.

Number of circulating leukocytes and haematological profiles of primiparous cows differed from that of pluriparous cows, particularly immediately before and after parturition. The elevated leukocytes and neutrophil counts during these periods may
reflect a response to the inevitable higher and short-lasting circulating levels of cortisol, a known marginal pool enhancer (Boggs et al., 1964), in periparturient dairy cows in both groups (Guidry et al., 1976; Peter and Bosu, 1987). Comparatively, the considerably higher circulating immature PMN in primiparous cows reveals that the overall haematopoiesis is more functional in primiparous dairy cows. This could explain the existence of more juvenile neutrophils in circulation, consequently boosting PMN viability and “extending their functional lifespan” in the milk compartment. The remarkably higher circulating eosinophils in primiparous cows during periparturition strongly suggest more active eosinophilopoiesis in primiparous cows. Though not as crucial as PMN in mastitis, the way in which compound I of eosinophil peroxidase reacts with H$_2$O$_2$ is similar to that of MPO, but with substrates like Cl$^-$, however, it is far higher (Arnhold et al., 2001), which yields the strongest bactericidal substance: HOCl. Moreover, micromolar of eosinophil major basic protein activates neutrophil in noncytotoxic manner, substantially boosting PMN CL (Moy et al., 1990). The involvement of eosinophil in bactericidal activity of bovine blood PMN especially during periparturient period still remains to be documented.

In short, the more pronounced PMN CL and milk PMN viability suppression during periparturient period in pluriparous cows can reflect PMN bactericidal inefficiency during the early phase of bacterial invasion, potentially boosting pluriparous cow’s susceptibility to mastitis. Whether the primiparous dairy cows’ udders are more protected against invading pathogens remains to be tested.

**Acknowledgements**

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**References**


Table 1. Mean differences (with 95% confidence interval) between primiparous and pluriparous cows during three different study periods: 1) PPD-3,-2: at 2 or 3 days before parturition, 2) PPD2,3,4: at 2.3 or 4 days after parturition, 3) PPD+7: from 7 to 35 days after parturition. For the third study period, intercept is related to the mean difference at day 20 after parturition, whereas the slope is related to the difference in the change of the parameter over time.

<table>
<thead>
<tr>
<th>Dependent variables</th>
<th>Primiparous vs. pluriparous</th>
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<tr>
<td></td>
<td>ppd -2,-3</td>
</tr>
<tr>
<td>Blood PMN CL*</td>
<td>3407 (2398; 4417)</td>
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<tr>
<td>Milk PMN CL*</td>
<td>469 (100; 839)</td>
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<tr>
<td>Blood PMN viability (%)</td>
<td>1.0 (0.0; 1.9)</td>
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<td>Milk PMN viability (%)</td>
<td>33 (28; 38)</td>
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<tr>
<td>SCC (×1000 / ml)</td>
<td>-38.8(-13.6; -63.9)</td>
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<tr>
<td>WBC / µl</td>
<td>3719(2377; 5061)</td>
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<tr>
<td>Mature neutrophils / µl</td>
<td>2604(2072; 3137)</td>
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<tr>
<td>Band cells / µl</td>
<td>113(78; 147)</td>
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<tr>
<td>Meta + Myelo / µl</td>
<td>604(363; 845)</td>
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<tr>
<td>Eosinophils / µl</td>
<td>613.6(456; 771)</td>
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<td>Blood PMN CL*</td>
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<td>Milk PMN viability (%)</td>
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<td>WBC / µl</td>
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<td>Mature neutrophils / µl</td>
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<td>Blood PMN viability (%)</td>
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<td>Milk PMN viability (%)</td>
<td>22 (16;27)</td>
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<tr>
<td>SCC (×1000 / ml)</td>
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<td>Eosinophils / µl</td>
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<td>WBC / µl</td>
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<td>Meta + Myelo / µl</td>
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<tr>
<td>Eosinophils / µl</td>
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* Area under the curve (AUC) of 1000 viable PMN
Figure 1. Chemiluminescence (CL) of PMA stimulated blood (a) and milk (b) PMN in primiparous (n = 12, solid line and circles) and pluriparous (n = 12, dashed line and triangles) dairy cows during periparturient period (0 indicates the day of parturition). Data are expressed as the area under the curve (AUC) of continuously light emission of $10^3$ viable PMN for 30 minutes.
Figure 2. Blood and milk PMN chemiluminescence profiles of primiparous and pluriparous dairy cows at day 2 before and day 2 after calving. The generation of CL was monitored continuously for 30 min after addition of 100 ng/ml PMA and 0.3 mM luminol to the $4 \times 10^5$ isolated cells suspended in 200 µl. The data shown are means of 5 primiparous and 5 pluriparous dairy cows.
Figure 3. Viability of blood (a) and milk (b) PMN in primiparous (n=12, solid line and circles) and pluriparous (n=12, dashed line and triangles) dairy cows during periparturient period (0 indicates the day of parturition). Values are expressed as percent of viable PMN.
Part 3

Studies performed during mastitis
Chapter 6

Local and systemic effects of endotoxin mastitis on the chemiluminescence of milk and blood neutrophils in dairy cows

This chapter is based on:

J. Mehrzad, H. Dosogne, E. Meyer and C. Burvenich

Veterinary Research (2001) 32: 131-144
Abstract

The local and systemic effects of intramammary lipopolysaccharide (LPS) injection on the chemiluminescence (CL) of milk and blood polymorphonuclear leukocytes (PMN) were investigated in six healthy early lactation cows. Clinical signs of acute mastitis such as fever, increased heart rate and a decreased milk production were observed in all cows. Before LPS challenge, CL activity of milk PMN was significantly lower than of blood PMN (P < 0.01). A significant negative correlation was found between pre-challenge milk and blood PMN CL and the decreased milk production in unchallenged quarters. CL activity of milk PMN from LPS-injected quarters increased following LPS challenge, whereas it remained unchanged in control quarters. CL activity of blood PMN showed a biphasic increase, with two peaks and a valley below pre-challenge CL activity (P < 0.01). At post-challenge hours (PCH) 6 and 12, the CL activity of milk PMN from LPS-injected quarters exceeded that of blood PMN (P < 0.05 and P < 0.001, respectively). The decreased CL activity of blood PMN and the enhanced CL activity of milk PMN during endotoxin-induced mastitis was reflected by changes in the shape of the CL curve. In blood PMN, a decrease of the second peak of the CL curve suggests that the myeloperoxidase (MPO)-H₂O₂ system is impaired during endotoxin-induced mastitis. In contrast, the MPO-H₂O₂ system was enhanced in milk PMN from challenged quarters. The highest duration and intensity of reactive oxygen intermediates (ROI) production was observed in milk PMN from LPS-injected quarters at PCH 12. The increased viability of PMN in LPS-injected quarters and to a lesser extent in control quarters suggests possible effects of both facilitated diapedesis and inflammatory mediators on milk PMN survival. In conclusion, our results suggest that a combination of local and systemic actions of *E. coli* endotoxin is involved in the priming of milk PMN during mastitis.
1. Introduction

Polymorphonuclear leukocytes (PMN) are important components of the cellular host defense to protect cows against *Escherichia coli* infection within the mammary gland (Burvenich et al., 1994; Heyneman et al., 1990; Jain et al., 1976; Kremer et al., 1993; Van Werven et al., 1997). Acute *E. coli* mastitis in early lactating cows is sometimes accompanied by severe general clinical symptoms such as fever and a sudden decrease of milk production in non-injected (non-inflamed) quarters (Hill et al., 1979; Lohuis et al., 1988; Vandeputte-Van Messom et al., 1993). Large quantities of endotoxin (lipopolysaccharide, LPS) are released during *E. coli* mastitis upon killing of bacteria, as well as during the exponential phase of bacterial growth (Carlson et al., 1973; Hill et al., 1979). Many effects of *E. coli* mastitis can be mimicked by its endotoxin suggesting that LPS plays an important role in the pathophysiology of coliform mastitis (Carlson et al., 1973; Hill et al., 1979; Ziv et al., 1982). The endotoxin mastitis model, however, is only partially comparable to the *E. coli* model. Because systemic symptoms following intravenous and intramammary injection of LPS differ, it has been suggested that endogenous mediators other than endotoxin are released and resorbed from the inflamed udder (Lohuis et al., 1988a; b). Nevertheless, the endotoxin model can be used to study the role of LPS in the pathogenesis of *E. coli* mastitis.

The intramammary defense against invading pathogens is highly dependent on the rate of PMN diapedesis into the infected quarters (Hill et al., 1979), their capacity to produce reactive oxygen intermediates (ROI; Heyneman et al., 1990) and the number of circulating PMN prior to infection (Heyneman et al., 1990; Kremer et al., 1993; Vandeputte-Van Messom et al., 1993; Van Werven et al., 1997). Chemiluminescence (CL) can be used to study the oxygen-dependent bactericidal system of PMN (Allen et al., 1972; Dulin et al., 1988; Leino and Paape, 1993; Mehrzad et al., 2000). In healthy cows, milk PMN could be considered as rather ineffective cells, compared to circulating PMN, because of their short half-life time (t 1/2 = ± 8h), the exhaustion of their intracellular glycogen reserve (Naidu et al., 1973), the potential induction of apoptosis and their decreased ROI generation following diapedesis (Smits et al., 2000). Although the CL of milk PMN is lower than their blood counterparts (Mehrzad et al. unpublished), the resident milk PMN could play an important role in the intramammary defense system against invading bacteria and their endotoxins because a significant influx of PMN from the circulation is not observed until 8h after infection. Therefore, in our study the pre-challenge CL activity of milk PMN was assessed in order to investigate the role of the resident PMN in milk in the pathogenesis of coliform mastitis.

During *E. coli* mastitis, the ROI production of blood PMN has been observed to decrease (Heyneman et al., 1990). The ROI production of milk PMN during mastitis, however, is not well characterized. The kinetics of ROI generation by blood and especially milk PMN during coliform mastitis has not been investigated. In addition, comparison of the ROI production of PMN from infected- and non-infected quarters can provide an insight into the local and systemic effects on PMN function during coliform mastitis. The viability and the maturity of PMN were also determined to provide insight in the observed alterations in CL activity.
2. Materials and Methods

This experiment has been approved by the ethical committee of the Faculty of Veterinary Medicine from the Ghent University.

2.1. Animals
Six cows of the East-Flemish Red pied breed in their first lactation and between 20 and 35 days after calving were used. These cows were selected on the basis of 2 consecutive bacteriologically negative milk samples and a milk somatic cell count (SCC) of <2.10^5/ml milk per individual quarter. Cows were transferred to the individual stalls 1 week before the start of the experiment. They were fed a daily ration of approximately 8 kg of concentrate and had free access to water and hay. They were milked twice daily at 7 a.m and 4 p.m with a 4-quarters milking machine.

2.2. LPS challenge
Ten mg LPS from *E. coli* O111: B4 (Sigma Chemical Co., St Louis, MO, USA) was diluted in 100 ml pyrogen-free 9 g/l saline solution and aliquoted in bottles of 5 ml LPS solution (500 µg in 5ml). All air was removed from the bottles by a sterile N2–flow. LPS solutions were stored at –20 °C until use. Frozen LPS solutions were thawed immediately before a challenge experiment and 15 ml of pyrogen-free saline solution was added. Before LPS injection the teat ends were disinfected with ethanol (70%) mixed with chlorohexidine. Endotoxin mastitis was induced – after the morning milking - into the left front and rear quarters by a single intramammary (i.mam) injection of 20 ml LPS solution per quarter (25 µg LPS/ ml, final concentration) using a sterile teat cannula. After injection, each quarter was massaged for 30 sec to distribute the LPS solution in the gland.

2.3. Milk and blood sampling
Quarter milk samples were aseptically collected for isolation and determination of somatic cell count (SCC) at 24 h before, immediately before and at 6, 12, 24, 48, 72, 144, 216 h following LPS injection. Peripheral blood (80 ml) was collected aseptically from each cow by venipuncture from the external jugular vein into evacuated tubes (Laboratory EGA, F-28210 Nogent-le Roi, France) containing 125 i.u. heparin as anticoagulant. The blood sampling was carried out subsequently after milk sampling at 24 h before, immediately before and at 6, 12, 24, 48, 72, 144, 216 h following LPS challenge.

2.4. Clinical symptoms
Measurements of rectal temperature (RT), heart rate (HR), rumen motility and clinical examination of the mammary gland were performed at the time of blood and milk sampling. Evening and morning milk were pooled to obtain daily milk production (MP). To assess the severity of LPS induced mastitis, the MP loss at post-challenge hours (PCH) 24 compared to the pre-challenge MP was used.

2.5. Cell counting in blood and milk
The total number of circulating leukocytes and isolated cells from blood and milk were determined using an electronic particle counter (Coulter counter ZF, Coulter Electronics Ltd., Luton, UK). Forty µl of whole blood or 25 µl of isolated cell suspensions were diluted in 20 ml of isotonic counting solution (Isoton® II, Counter Electronics, D-47705 Krefeld, Germany). Two hundred microliters and 100 µl,
respectively, lysis solution (containing (g.L⁻¹) KCN 3.3, sodium nitroprusside 1.1, quaternary ammonium salt 55; Zap-Oglobin®; Coulter Electronics) was added to the suspensions of whole blood and isolated blood cells. No lysis solution was used for the isolated milk cells. Determination of the cell number was performed with optimized instrument settings using a dual threshold model. The lower size threshold (Tl) for whole blood samples was set at 3 µm, whereas the Tl for isolated cells from milk and blood was set at 5 µm. The size of cells between Tl to 12 times Tl was used for quantification of cells in the sample. Five hundred microliters of blank agent and the cell suspensions were entered in the counter. The counting procedure was performed in triplicate, the value of the blank was subtracted for each sample and the mean was calculated as the number of cells.mL⁻¹ of the original sample. The SCC was determined for each individual quarter by a Fluoro-optoelectronic cell counting procedure (Fossomatic® 360; Foss Electronic, Eden Prairie, MN, USA).

2.6. Isolation of PMN from milk
Individual quarter milk samples (200 mL) were collected aseptically and subsequent isolation of cells was performed according to Dulin et al., (1988) with minor modifications. Briefly, pooled milk of the 2 LPS-injected and the 2 non-injected quarters of each cow was filtered separately through a nylon filter (40µm pore size) and diluted to 50% v/v with cold phosphate buffered saline (PBS; 0.01 M – Phosphate (KH₂PO₄-Na₂HPO₄)–0.15 M- NaCl, pH = 7.2). Fat was carefully removed after the first centrifugation (600 × g, 15 min, 4°C), and the pellet was washed twice in cold PBS and centrifuged at 300 ×g for 10 min and 200 × g for 15 min at 4°C. The cells were resuspended in Dulbecco’s PBS (Gibco BRL, Life Technologies Inc., MD Gaithersburg, USA) supplemented with 0.5 mg.mL⁻¹ gelatin (DPBSG). After counting, the viability and % of PMN of the isolated milk cells was determined (see below), and the cells were finally resuspended in a concentration of 5.10⁶ viable PMN mL⁻¹ in DPBSG solution.

2.7. Isolation of PMN from blood
Isolation of PMN from peripheral blood was performed using hypotonic lysis of erythrocytes according to Carlson and Kaneko, (1973). After counting, the viability and % of PMN of the isolated blood cells were determined (see below), and the cells were finally resuspended in a concentration of 5.10⁶ viable PMN mL⁻¹ in DPBSG solution.

2.8. Differentiation and viability of cells
Differential cell counts on the isolates were performed on eosin-Giemsa (Hemacolor®, Merck, Germany) stained smears. Identification of the milk cells was based on morphological characteristics as described by McDonald and Anderson, (1981). PMN were characterized by their multi-lobed or sometimes picnotic dark-bluish stained nucleus. Macrophages (M) typically had a large size, a vacuolated nucleus and contained whitish fat globules in their cytoplasm. Lymphocytes (L) were either large with a low nucleus-cytoplasm-ratio and light blue stained nucleus or small with a high nucleus-cytoplasm-ratio and a dark-bluish stained nucleus. Epithelial cells (only in milk) were identified as large, polygonal, uniformly light bluish stained cells. Differential leukocyte counts were also performed with light microscopy on whole blood and isolated blood cell smears stained with eosin-Giemsa as described by Hayhoe and Flemans, (1969). PMN (both mature and immature), M, L and epithelial cells were identified on at least 200 cells per slide. The viability of PMN in isolated
cells, both from blood and milk, was determined using trypan blue dye exclusion according to Colotta et al. (1992).

2.9. Chemiluminescence assay
Luminol-enhanced PMA (phorbol 12-myristate, 13-acetate)-stimulated cellular chemiluminescence (CL) was used to measure the CL activity of cells isolated from blood and milk of LPS-injected and non-injected quarters. Briefly, the CL was measured in duplicate during 30 min at 37 °C with a microtiterplate luminometer (type LB96P; EG&G Berthold, D-75312 Bad Wildbad, Germany), immediately after addition of 100 ng.mL⁻¹ PMA and 0.3 mM luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione, Sigma) to 2.10⁶ cells.mL⁻¹ in a total volume of 200 µl per well. Stock solutions of PMA and luminol were prepared in dimethyl sulphoxide (DMSO) (Sigma) and always stored at – 20 °C. The area under the curve (AUC) was calculated for the registered impulse rates (relative light unit (RLU).s⁻¹) over the whole measurement period of 30 min. The CL response was expressed per 10^3 viable PMN in each isolated cell sample.

The following formula was used to perform the corrections, where \( \text{Cl} = \text{mean RLU.s}^{-1} \), 4.10⁵=total number of cells per well, \% PMN=total percentage of PMN in isolated cells, \% V=percentage of viable PMN:

\[
\text{CL}_{\text{PMN}} = \frac{\text{Cl}_{\text{isolated cells}} \times 10^3}{4.10^5 \times \% \text{PMN} \times \% V}
\]

The CL of blood PMN was calculated with the same formula as for milk PMN applying the corrections described by Heyneman et al. (1990) for eosinophils contributing to the ROI production in the samples, considering that the CL response of eosinophils is five times higher than that of PMN.

2.10. Shape of the CL curve
To assess the kinetics of ROI generation during endotoxin mastitis, the profiles of cellular ROI production of milk and blood PMN were performed before challenge (-24 h) and at PCH 12 in all cows.

2.11. Statistical analyses
The Statistix program package (version 4.1, Analytical Software, Tallahassee, FL, USA) was used for the statistical analyses. Because the values of PMN characteristics following injection of LPS were not normally distributed, a Kruskal - Wallis test was used to compare blood and milk PMN characteristics and the effects of LPS mastitis. The time of sampling was a fixed factor, cows were a randomized factor, and their interaction term was the error term. Significance of the differences was determined at \( P < 0.05^* \), \( P < 0.01^{**} \) and \( P < 0.001^{***} \).

Correlations between blood and milk PMN CL before LPS challenge, and between MP loss in non-challenged quarters and PMN functions before challenge was assessed using linear regression.
3. Results

3.1. Clinical observations
Intramammary administration of LPS induced an increase of RT and HR that peaked around PCH 6, as well as swelling and pain of the challenged quarters, appearance of flecks and milk leakage in LPS injected quarters observed at PCH 3 to 6 (data not shown) for all cows. The MP was reduced with 49 ± 6 % in non-injected quarters and with 80 ± 6 % in LPS-injected quarters at PCH 24. It was completely restored at PCH 72 and 96 in non-injected and injected quarters, respectively (Figure 1). Between PCH 6 and 12, a sharp increase of SCC was observed in LPS-injected quarters. At PCH 12 the SCC value exceeded the maximal detection capacity of the method (Figure 2). The SCC in control quarters was not changed. Clinical signs of mastitis disappeared around PCH 72.

3.2. Differentiation of PMN in isolated milk cells
Before LPS injection, the percentage of PMN in isolated milk cells was 57 ± 2%. Compared to pre-challenge values, the % of milk PMN increased significantly at PCH 6 and 12 in LPS-injected quarters (P < 0.001) and to a lesser extent in non-injected quarters (P < 0.05) (Table 1). In non-injected quarters the % of PMN returned to pre-challenge values at PCH 72. The % of milk PMN in LPS-injected quarters also decreased, but remained higher than pre-injection values (P < 0.01) until 216 h after challenge.

3.3. Number and differentiation of blood cells
Leukopenia was observed between PCH 6 and 12 reaching a minimum at PCH 6 (Figure 2). This was followed by a leukocytosis from PCH 24 to 48. At PCH 6, band cells appeared in the circulation and a peak of immature neutrophils, which was 47 % of total isolated neutrophils, was seen at PCH 12 (Table 1). Metamyelocytes were observed in the circulation at PCH 6 to 48 (Table 1). A positive correlation was found between the % of immature neutrophils in whole blood and in isolated blood cells (r = 0.92; P < 0.001; n = 56).

3.4. CL of milk and blood PMN
Compared to pre-challenge values, the CL activity in isolated milk PMN from LPS-injected quarters increased with 28 ± 4 % and 37 ± 4 % (P < 0.001) at PCH 6 and 12, respectively (Figure 3). At PCH 6 and 12, the CL activity of milk PMN superseded that of blood PMN (P < 0.05 and P < 0.001, respectively). The CL response of PMN isolated from LPS-injected quarters was higher than of non-injected quarters at PCH 6, 12 and 24 (P < 0.001, P < 0.001 and P < 0.05), respectively. Pre-challenge values were again obtained at PCH 48 in LPS-injected quarters. The CL activity of isolated milk PMN of non-injected quarters did not change except for a slight increase at PCH 12. Before LPS challenge, the CL activity of blood PMN was higher than of milk PMN (P < 0.01). The CL activity of PMN isolated from blood showed a biphasic increase following LPS challenge, with peaks at PCH 6 and 72 (Figure 3). At PCH 12 the CL activity was lower than before challenge time (P < 0.01). The valley between both CL activity peaks in blood corresponded to the dramatic increase observed in the CL activity of milk PMN from LPS injected quarters (Figure 3). A negative correlation was found between pre-challenge CL activity of milk and blood PMN and
MP loss in non-injected quarters \((r = -0.86, P < 0.05\) and \(r = -0.87, P < 0.05\), respectively; \(n = 6\)\) (Table 2). A significant positive correlation was found between pre-challenge CL values of milk and blood PMN \((r = 0.76; P < 0.01; n = 30)\).

### 3.5. Shape of CL curves from blood and milk PMN

Figure 4 shows representative CL profiles after stimulation with PMA of both blood and milk PMN at \(-24\) h (Figure 4a), and at PCH 12 (Figure 4b) following PMA stimulation. The ROI production in blood followed a typical biphasic pattern both before and after endotoxin mastitis with peaks after 4 and 12 minutes. However, at PCH 12 the CL peaks were lower, which was more pronounced at the second peak.

The intensity and duration of milk cell CL from LPS-injected quarters increased markedly at PCH 12 in comparison with the pre-challenge values. In contrast to the pre-challenge values, the intensity and the duration of ROI production of milk cells from non-injected quarters was also increased. The CL of milk cells from inflamed quarters was higher than of blood cells and of milk cells isolated from non-injected quarters at PCH 12. Although the intensity of ROI production was higher, milk cells never demonstrated the biphasic pattern of the CL curve, which was typical for blood PMN.

### 3.6. Viability of milk and blood PMN

Before LPS challenge, the viability of PMN isolated from milk was \(54 \pm 1\%\). The viability of PMN isolated from milk of both LPS-injected and non-injected quarters increased at PCH 6 to 92 and 85%, and at PCH 12 to 94 and 82% \((P < 0.001)\), respectively (Figure 5). Viability was higher in PMN from LPS-injected quarters than from non-injected quarters \((P < 0.01)\). Pre-challenge values were again obtained at PCH 216. The viability of PMN isolated from blood varied between 96 and 97% and did not change significantly during the experiment (Figure 5).

### 4. Discussion

In the present study both local and systemic aspects of the nonspecific inflammatory response of the bovine mammary gland of early lactating cows were investigated in an acute endotoxin-induced mastitis model. This model was chosen because it mimics many responses occurring during natural \(E\). \(coli\) mastitis (Carroll et al., 1964; Hill et al., 1979; Ziv et al., 1982). Acute mastitis caused by LPS administration provoked local as well as systemic effects: inflammation of the LPS injected quarters with an increase of SCC, decreased MP, fever and increased TPR (temperature, pulse and respiration). The lactating gland of the dairy cow is known to be sensitive to endotoxin with very low doses producing an inflammatory response (Schultze et al., 1970; Frost et al., 1984; Shuster et al., 1991). It was not surprising therefore, to observe a severe local reaction and clinical symptoms by higher doses of LPS as in our study. The magnitude of decreased MP, particularly in non-injected quarters, reflects the extent of systemic illness as demonstrated by Burvenich and Peeters, (1983) in lactating goats, and by Shuster et al. (1991) in lactating cows. In spite of observed systemic effects of LPS, it remains unclear which molecule(s) is/are directly involved in the uncontrolled pathogenesis in severely diseased cows during \(E\). \(coli\) mastitis. Several recent studies suggest an important role of tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) in mediating the severity of the clinical symptoms during \(E\). \(coli\) mastitis (Blum et al., 2000; Hirvonen et al., 1999). Milk production is undoubtedly related to the normal functionality of the mammary epithelium. However, LPS itself has no
direct damaging effects on mammary epithelium (Frost et al., 1984). Therefore, the decreased MP observed in inflamed quarters must have been caused by other than a direct effect of LPS.

In this study a negative correlation was found between the pre-challenge ROI generation of milk PMN and MP loss in non-injected quarters. A possible explanation for this observation is that milk PMN play a role in the direct detoxification of LPS by ROI production at the site of LPS injection. Consequently, LPS would be less available to initiate an inflammatory response and subsequent induction of agalactia in non-injected quarters. Similarly, the ROI generation of blood PMN was as important as their milk counterparts. Blood PMN respiratory burst activity is indeed important for an efficient elimination of intramammary pathogens or LPS after migration into the mammary gland, as previously demonstrated in an *E. coli* mastitis model (Heyneman et al., 1990). In addition, the detoxification of LPS by blood PMN could be important for prevention of a severe systemic response to endotoxin.

Many host-derived cytokines such as interleukin-1 (IL-1), IL-6 (Shuster et al., 1993) and TNF-α (Hoeben et al., 2000) are produced during LPS induced mastitis. They cause systemic effects such as fever and increase the bone marrow output of leukocytes to compensate for the decrease in the circulating pool (Goff et al., 1992). Consequently, there is a transient increase in the circulation of the number of immature neutrophils as also observed in the present study. The host-derived cytokines also induce an increased capillary permeability, an increased permeability of the blood/milk barrier and a concomitant exudation of proteins such as bovine serum albumin (BSA) in lacteal secretions (Kehrli et al., 1994). Increased permeability of the blood/milk barrier was indicated by the appearance of clots in milk at PCH 6. Between PCH 6 and 12 a large number of PMN having accumulated in lacteal secretion of inflamed quarters resulted in a substantial increase of the SCC. The recruitment of PMN into the inflamed quarters, as reflected by the SCC (Kehrli et al., 1994), together with their increased ROI production are both important aspects of the defense mechanism against LPS during Gram-negative coliform mastitis.

The increased milk SCC in LPS-injected quarters coincided with an increased percentage, CL activity and viability of milk PMN, as well as a maximal decline of MP. At first sight, these phenomena could be explained by the enhanced migration of circulating PMN, resulting in a milk PMN viability and CL activity comparable to that of blood PMN. However, the CL activity of milk PMN exceeded that of blood PMN, suggesting an increased effectiveness of milk PMN upon accumulation in the inflamed udder following LPS exposure. According to Hoeben et al. (2000), milk levels of host-derived cytokines such as TNF-α are higher than blood levels between PCH 4 and 12. Therefore, TNF-α might be involved in the observed increase in the milk PMN CL. Our observation is in agreement with the results of Watson et al. (1997) who demonstrated that rat PMN isolated from LPS-treated bronchoalveolar lavage, showed a higher CL activity than circulating PMN.

The CL activity of isolated blood PMN was significantly increased at PCH 6. This increase could be due to priming effects of increased plasma concentrations of cytokines such as IL-1 and IL-6 in response to LPS (Shuster et al., 1993). Hoeben et al. (2000) demonstrated that plasma TNF-α levels maximized at 4 to 8 h following intramammary injection with LPS. Indeed, IL-1 and TNF-α can both prime
circulating PMN to produce ROI following activation with PMA (Ferrante, 1992; Sample and Czuprynski, 1991; Wewers et al., 1990). The second increase of the CL activity of blood PMN at PCH 72 could be due to the production of additional inflammatory mediators priming the newly released PMN from the bone marrow. It remains unclear which molecules are responsible for an enhanced ROI production of these PMN.

A decrease of the CL activity of circulating PMN was observed at PCH 12. Similar results were observed at PCH 18 during induced *E. coli* mastitis (Heyneman et al., 1990; Van Oostveldt et al., 1999). This delay of 6 h in the decrease of respiratory burst activity of circulating PMN in the *E. coli* mastitis model with respect to the LPS model can be explained by the differences in the inflammatory response between endotoxin and *E. coli* challenge. The decreased blood PMN CL activity at PCH 12 was accompanied by an increased % of band neutrophils, metamyelocytes and myelocytes in circulation. This is similar to observations during *E. coli*-induced mastitis and can be ascribed to the indirect effect of LPS on the release of immature neutrophils from the bone marrow. The decreased respiratory burst activity of circulating PMN at PCH 12 can be explained by this increased immaturity. Indeed, immature PMN have a lower ROI production (Glasser and Fiederlein, 1987; Heyneman et al., 1990).

The profiles of cellular ROI production before challenge and at PCH 12 showed remarkable differences. In general, the changes during endotoxin-induced mastitis were different in PMN from blood and from milk of challenged and non-injected quarters. More specifically, the changes in blood and milk PMN were reversed, with a decrease in the CL curve of blood PMN and an increase in that of milk PMN. The decreased intensity of ROI production of blood PMN was more pronounced at the second peak of the CL profile. According to Briheim et al. (1984), both intra- and extracellular events contribute to the luminol-dependent CL of human PMN. The first peak of the CL curve is probably the result of the initial extracellular reactions, whereas the second peak is the result of further intracellular reactions of the MPO-H$_2$O$_2$ system. Thus, our findings suggest that the observed decrease of PMN CL activity during endotoxin-induced mastitis is primarily due to a decrease activity of the MPO-H$_2$O$_2$ system. This could be explained by immaturity of neutrophils as the MPO enzyme is not present in myeloblasts and immature neutrophils have a decreased MPO activity. The increased intensity and maximal duration of ROI production in the CL profile of milk PMN from inflamed quarters at PCH 12 suggests an increase of both intra- and extracellular ROI production. These effects could be explained by activation of mature PMN caused by local production of inflammatory cytokines. Compared to inflamed quarters, the less strongly increased intensity and duration of ROI production of PMN from non-injected quarters, however, indicates that primed blood PMN may have been diapedesed into non-injected quarters. This suggests that inflammatory mediators were systemically released, leaking into the non-injected quarters, thereby possibly priming PMN for the slightly enhanced CL activity. In conclusion, it appears that the local production of inflammatory mediators is responsible for priming of PMN between PCH 6 and 12 during endotoxin-induced mastitis, enabling milk PMN to exhibit a maximal bactericidal efficacy. The remarkable differences in the kinetics of milk and blood PMN function during mastitis require further study.
The viability, as opposed to necrosis, of milk PMN of LPS-injected quarters increased significantly between PCH 6 and 12. The modulation of bovine PMN survival in milk by LPS could be linked to the pathophysiology of endotoxin-induced mastitis, especially the kinetics of PMN diapedesis through the blood/milk barrier. As seen in our study, LPS induces a rapid migration of circulating PMN into the mammary gland. It also stimulates PMN diapedesis through the blood/milk barrier (Paape et al., 1996). Indeed, LPS directly or indirectly (Ferrante, 1992) induces adherence of circulating PMN to the endothelium by up-regulation of CD11b/CD18 (Roets et al., 1999). The CD11b/CD18 molecule is crucial to bovine PMN diapedesis across the blood/milk barrier (Smits et al., 2000). In addition, the increased concentrations of BSA in milk from challenged quarters suggest an increased permeability of the blood/milk barrier. The enhanced diapedesis across the blood/milk barrier in combination with the increased permeability of this barrier could result in a significantly higher viability of milk PMN from LPS-injected quarters. Beyond this initial fast PMN influx into inflamed quarters, both LPS and some host-derived cytokines are known to delay apoptosis and subsequently increase viability of circulating PMN (Colotta et al., 1992; Lee et al., 1993). Watson et al. (1997) observed a delayed apoptosis and increased viability of PMN during LPS induced migration in the lung of the rat. A less pronounced increase of milk PMN viability was observed in the non-injected quarters. Once again because of the absence of initial local mammary inflammation in the non-injected quarters, this increased viability of PMN was most likely caused by systemic effects of LPS.

In conclusion, the strong enhancement of PMN CL activity observed in the LPS-challenged quarters is a consequence of both enhanced migration of blood PMN into the mammary gland and priming by locally produced inflammatory mediators. The correlation between pre-challenge CL activity of the resident PMN in milk and the decrease of MP after LPS challenge suggests that the ROI production of milk PMN could play an important role in local neutralization of LPS and the subsequent inflammatory response prior to a significant influx of PMN in milk. Together with the correlation between blood and milk PMN CL activity, our findings suggest that low milk PMN CL activity could be considered as a risk factor for severe clinical coliform mastitis.

**Acknowledgements**

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polymorphonuclear leukocytes to predict severity of *Escherichia coli* mastitis. J. Dairy Sci. 76: 1568-1574.


Table 1. Percentage of isolated milk PMN from LPS-injected and non-injected quarters, of PMN and of immature neutrophils (sum of band cells, myelocytes and metamyelocytes) isolated from blood. Values are the means ± SEM of 6 cows. The level of significance is indicated with asterisks (*: P < 0.05, **: P < 0.01 and ***: P < 0.001). Time 0 = day of LPS challenge.

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<td>55 ± 1.1</td>
<td>56 ± 0.9</td>
<td>82 ± 4</td>
<td>0.2 ± 0.1</td>
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<tr>
<td>0</td>
<td>60 ± 0.6</td>
<td>60 ± 1.5</td>
<td>83 ± 4</td>
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<tr>
<td>6</td>
<td>92 ± 0.7**</td>
<td>71 ± 0.7*</td>
<td>71 ± 4*</td>
<td>10 ± 1*</td>
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<tr>
<td>12</td>
<td>98 ± 0.8***</td>
<td>69 ± 0.9*</td>
<td>42 ± 8***</td>
<td>38 ± 9***</td>
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<tr>
<td>24</td>
<td>89 ± 0.9**</td>
<td>64 ± 1.3</td>
<td>76 ± 7</td>
<td>13 ± 5**</td>
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<tr>
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<td>65 ± 2.1</td>
<td>78 ± 4</td>
<td>8.0 ± 2*</td>
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<td>74 ± 1.4**</td>
<td>62 ± 1.3</td>
<td>88 ± 2</td>
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<tr>
<td>144</td>
<td>73 ± 2.2**</td>
<td>55 ± 3.0</td>
<td>92 ± 2</td>
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<tr>
<td>216</td>
<td>73 ± 2.3**</td>
<td>56 ± 2.6</td>
<td>91 ± 1</td>
<td>0.4 ± 0.2</td>
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Table 2. Correlation between CL activity of milk and blood PMN, WBC and SCC averaged prior to LPS-challenge (d –4 to d 0) and milk production loss in control quarters at d +1 of the LPS challenge.

<table>
<thead>
<tr>
<th>Pre-challenge parameter</th>
<th>r</th>
<th>Statistical significance</th>
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<tr>
<td>CL of milk PMN</td>
<td>-0.86</td>
<td>P = 0.018</td>
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<tr>
<td>SCC</td>
<td>-0.21</td>
<td>NS^a</td>
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<tr>
<td>CL of blood PMN</td>
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<td>P = 0.017</td>
</tr>
<tr>
<td>WBC</td>
<td>-0.37</td>
<td>NS^a</td>
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</table>

^a not significant
Figure 1. Daily milk production (liters / udder halve) from the LPS-injected (■) and non-injected (□) quarters of cows during LPS mastitis. Values are the means ± SEM of 6 cows. Time 0 = day of LPS challenge.
Figure 2. Changes in somatic cell count (SCC) of LPS-injected (■) and non-injected (□) quarters and in total WBC (▲) of cows during experimentally induced LPS mastitis. Data are means ± SEM of 6 cows.
Figure 3. Chemiluminescence of stimulated milk PMN isolated from LPS-injected (■) and non-injected (□) quarters and from blood (▲) of cows during experimentally induced LPS mastitis. Values are the mean of 6 cows and error bars represent SEM.
Figure 4. Chemiluminescence profile of stimulated-isolated blood and milk cells at 24 h before (a) and 12 h after experimentally induced LPS mastitis (b). Values are the mean of 6 cows and error bars represent SEM.
Figure 5. Viability of milk PMN isolated from LPS-injected (■) and non-injected (□) quarters and of blood (▲) of cows during experimentally induced LPS mastitis. Values are the mean of 6 cows and error bars represent SEM.
Chapter 7

Induction of clinical *Escherichia coli* mastitis in dairy heifers: Effect on respiratory burst activity and viability of blood and milk neutrophils
Abstract

To study the changes of blood and milk polymorphonuclear leukocytes (PMN) reactive oxygen species (ROS) load and viability during *Escherichia coli* (*E. coli*) mastitis, PMA and latex stimulated and non-stimulated chemiluminescence (CL) of blood and milk PMN was followed up in early lactation cows after intramammary *E. coli* inoculation. Clinical signs of acute mastitis were observed in all cows with maximal milk somatic cell count (SCC) and colony forming units (cfu) in infected quarters appearing at post infection hours (PIH) 6-12 and 18-24, respectively. Pre-infection milk PMN CL was significantly lower than blood PMN CL. However, CL activity of PMN from *E. coli* infected quarters significantly increased following *E. coli* challenge, even exceeding to blood PMN CL at PIH 6 and 12. No such an increase was observed in control quarters. Similar results were obtained for PMA and latex stimulated PMN CL. The earlier decrease of PMA- and latex- stimulated CL of blood PMN and the enhanced CL of milk PMN during *E. coli* mastitis was reflected by the shape of the CL kinetics, resulting mainly from changes in PMN myeloperoxidase (MPO) and phagocytosis activity. The highest durability and intensity of ROS production was observed in milk PMN from infected quarters at PIH 12. Viability of PMN in infected quarters significantly increased at PIH 6 to 72 whereas a significant increase in the control quarters was only observed at PIH 6. This observation proves that the effects of coliform mastitis in heifers are predominantly local and to a lesser extent systemic. The higher PMN CL and rapid increase of PMN viability in infected quarters resulted mainly from fast PMN diapedesis into the infection sites. This might be a decisive factor in the elimination of *E. coli* from the mammary gland and thus prevent prolonged mastitis in primiparous dairy cows.
E. coli mastitis and its effect on PMN respiratory burst activity and viability

Introduction

Few infectious diseases in early lactating dairy cows are as important as mastitis (De Graves and Feltrow 1993) as acute Escherichia coli (E. coli) mastitis in early lactating cows is accompanied by severe clinical symptoms (Hill et al., 1979; Vandeputte-Van Messom et al., 1993). Polymorphonuclear leukocytes (PMN) are crucial components of the cow’s first line defense to both prevent and protect the udder from inflammation caused by E. coli (Heyneman et al., 1990; Kremer et al., 1993; Burvenich et al., 1994; Kehrli et al., 1994). Intramammary defense against invading pathogens heavily relies on the number of circulating PMN before infection, the rate of PMN diapedesis into the infected quarters (Hill et al., 1979; Kremer et al., 1993; Vandeputte-Van Messom et al., 1993) and their capacity to produce reactive oxygen species (ROS) (Heyneman et al., 1990). Also PMN viability, which is evidently influenced by the physiopathological conditions of the gland (Piccinini et al., 1999; Mehrzad et al., 2001a; b) is an important parameter in this defense mechanism.

PMN ROS can be quantified following PMN stimulation with soluble agents (e.g. PMA) or with particles (e.g. zymosan, bacteria, latex beads). The chemiluminescence (CL) assay is the most widely used technique to investigate the oxygen-dependent bactericidal system of PMN (Allen et al., 1972; Weber et al., 1983; Piccinini et al., 1999; Grebner et al., 1977). Throughout the lactation cycle, PMA-stimulated milk PMN CL in healthy cows varies from 47 to 78% of their blood counterparts (Mehrzad et al., 2001a). This is probably due to their short half-life time (t 1/2 < 8h), the exhaustion of their intracellular glycogen reserve (Naidu et al., 1973), PMN apoptosis (Van Oostveldt et al., 2002) and their ROS generation depression after diapedesis (Smits et al., 1999). However, the ratio of milk to blood PMN CL during E. coli mastitis could increase as seen during endotoxin mastitis (Mehrzad et al., 2001b).

During E. coli mastitis, the ROS production of blood PMN has been observed to decrease (Heyneman et al., 1990). The viability of blood PMN is known to be around 100% (Mehrzad et al., 2001b). How the ROS production and viability of milk PMN during E. coli mastitis evolves, however, is largely unknown. Whether differences between PMN of infected and non-infected quarters as observed in endotoxin mastitis (Mehrzad et al., 2001b) exist in E. coli mastitis is also debatable. Obviously, blood PMN recruit into both E. coli infected and non-infected quarters, of which assessment would enable us to study the physiopathology of E. coli mastitis and to distinguish between local and systemic effects. Thus, comparison of milk PMN CL and viability after normal diapedesis and accelerated diapedesis with those of blood offers additional details about host-pathogen interactions during E. coli mastitis.

To examine if there is any parallel between blood and milk (infected and non-infected quarters) PMN function before, during and following E. coli mastitis, the effect of E. coli infection on the PMN CL and viability was assessed. PMN maturity during E. coli mastitis was also determined to provide insight into the observed changes in PMN CL and viability. The kinetics of blood and milk PMN CL during highest PMN CL value was further studied in detail.
Materials and methods

This experiment has been approved by the ethical committee of the Faculty of Veterinary Medicine of Ghent University.

Animals and experimental procedures

Fifteen Holstein-Friesian Red pied breed cows in their 221±5 days of first pregnancy (2.3±0.2 yr) on arrival at the dairy farm (CDFO- Commercial Dairy Farm Oudenaarde, Oudenaarde, Belgium) were studied. The animals, on a zero-grazing system from arrival till the end of the experiment, were put to an individual stall and were fed with a special ration for pregnancy and lactation. They always had free access to water and hay. After gestation, clinically healthy ones, showing no sign of typical periparturient diseases, were eventually selected (n=15) on the basis of 2 consecutive bacteriological negative milk samples and a milk somatic cell count (SCC) of <2.10⁵/ml milk per individual quarter. One week before the start of the experiment the animals were fed a daily ration of approximately 8 kg of concentrate and had free access to water and hay. They were milked twice daily at 0800 and 1700 hours with a 4-quarters milking machine. *E. coli* was inoculated in the udder of animals at 20±5 days after parturition.

Individual quarter milk samples were aseptically collected for determination of cfu (10 ml), somatic cell count (SCC) (50 ml) and isolation (200 ml) at 24 h before, immediately before and at 6, 12, 24, 48, 72, 144, 216 and 312 h following *E. coli* injection. For bacteriological examination of milk, 0.5 ml of quarter milk was serially diluted in a pyrogen free saline solution (0.9%) and 0.01 ml of these diluted samples were streaked in duplicate on Columbia Sheep Blood Agar (Biokar Diagnostics, Beauvois, France) plates, using an inoculation loop. The plates were incubated for 24 h at 37 °C for cfu enumeration of the milk samples. Peripheral blood (80 ml) was collected aseptically from each cow by venipuncture from the external jugular vein into evacuated tubes (Laboratory EGA, F-28210 Nogent-le Roi, France) containing 125 i.u. heparin as anticoagulant. The blood sampling was carried out subsequently after milk sampling at 24 h before, immediately before and at 6, 12, 24, 48, 72, 144, 216 and 312 h following *E. coli* injection. Measurements of rectal temperature (RT), heart rate (HR), rumen motility and clinical examination of the mammary gland were performed at the time of blood and milk sampling. Evening and morning milk was pooled to obtain daily milk production (MP).

Bacterial challenge

*Escherichia coli* P4:032 isolated from a clinical case of mastitis was used. The stock of *E. coli* was maintained in lyophilized medium at –20 °C until use and frequently controlled for viability and purity. To prepare the inoculum, the bacteria were subcultured in brain-heart infusion broth (CM225; Oxoid, Nepean, ON, USA) at 37 °C. The bacterial suspensions were washed 3 times with pyrogen free saline solution (0.9%) and resuspended in the solution. Bacterial counting was performed using the plate count method in order to obtain the desired concentration. Before *E. coli* injection the teat ends were disinfected with ethanol (70%) mixed with 0.5% chlorohexidine. Twenty minutes after the morning milking *E. coli* mastitis was induced into the left udder half by a single intramammary (i.mam) inoculation of 10 ml of 10⁵ cfu *E. coli* P4:032 solution per quarter using a sterile teat cannula (7cm;
E. coli mastitis and its effect on PMN respiratory burst activity and viability

Me. Ve. Mat., Deinze, Belgium). After inoculation, each quarter was gently massaged for 30 seconds to distribute the bacterial solution in the gland.

**Blood and milk PMN preparation and enumeration**

All materials and reagents used for the isolation of blood and milk PMN were sterile. Isolation of PMN from peripheral blood was performed using hypotonic lysis of erythrocytes. The isolation procedure of PMN from blood yielded > 98% of granulocytes (PMN + eosinophils) with predominantly PMN (> 85%). After counting the cells using an electronic particle counter (Coulter counter Z2, Coulter Electronics Ltd., Luton., UK) and determining the viability (see below) and percentage of PMN, the cell suspensions were adjusted to a concentration of 5.10⁶ cells/ml in Dulbecco’s phosphate buffered saline (DPBS; Gibco BRL, Life Technologies Inc., MD, USA) supplemented with gelatin (0.5 mg/ml; Merck, Darmstadt, Germany). Individual quarter milk samples were used for subsequent PMN isolation as described previously (Mehrzad et al., 2001b). Briefly, pooled milk of the 2 E. coli-infected and the 2 non-infected quarters of each cow was filtered separately through a nylon filter (40µm pore size) and diluted to 60% v/v with cold DPBS. Isolation of PMN from milk was performed using three centrifugation steps as previously described (Mehrzad et al., 2001a; b). The total number of leukocytes and isolated blood and milk cells were determined using an electronic particle counter (Mehrzad et al., 2001a). The total number of different circulating leukocytes was determined using smear preparations of blood samples (Mehrzad et al., 2001a). Differential cell counts and staining procedures were performed on whole blood similar to the isolates on eosin-Giemsa-stained smears, using light microscopy. Cell identification was based on morphological characteristics as described by (Hayhoe and Flemans, 1969; McDonald and Anderson, 1981; Mehrzad et al., 2001a). To quantify percentages of each cell type in the samples, 200 cells per slide were classified as PMN (mature and immature), monocytes/macrophages, lymphocytes, eosinophils or epithelial cells (only in milk).

**Viability of milk and blood PMN**

The viability of isolated PMN from blood and milk (infected and non-infected) quarters was determined in duplicate by means of flow cytometry (FACSScan, Becton Dickinson Immunocytometry Systems, San José, CA, USA), using propidium iodide exclusion (Mehrzad et al., 2001a). The viability of PMN isolated from blood and milk of E. coli-infected and non-infected quarters were measured at PIH 6, 12, 18, 24, 48, 72, 144, 216 and 312.

**ROS production of milk and blood PMN**

PMN ROS production was quantified using CL assay; luminol-amplified PMA (phorbol 12-myristate, 13-acetate)-and-latex beads (polystyrene 0.76 µm diameter, 4.10¹¹ particles/ml; Sigma)-stimulated cellular CL was applied for CL of PMN isolated from blood and milk of E. coli-infected and non-infected quarters. CL was measured in duplicate during 30 minutes at 37°C with a microtiter plate luminometer (type LB96P; EG&G Berthold, D-75312 Bad Wildbad, Germany). PMA-stimulated CL was measured immediately after addition of 100 ng/ml PMA and 0.3 mM luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione, Sigma) to 2.10⁶ cells/ml in a total volume of 200µl per well. Similar concentrations of luminol and cells per well was used for latex beads (final concentration of 500 particles/PMN) stimulated CL. Stock solutions of PMA and luminol were prepared in dimethyl sulphoxide (DMSO) (Sigma) and always stored at – 20°C. The area under the curve (AUC) was calculated.
for the registered impulse rates (relative light unit (RLU)/s) over the whole measurement period of 30 minutes. The CL response was expressed per $10^3$ viable PMN in each isolated cell sample. Since the contribution of milk macrophages to luminol-dependent CL is negligible (Mehrzad et al., 2001a), the CL response was expressed per $10^3$ viable PMN and obtained as follows

$$CL_{MILK-PMN} = \frac{10^3 \times CL_{MEASURED}}{N_{CELLS} \times \% PMN \times \% VIAB}$$

with $CL_{MEASURED}$ the measured chemiluminescence

$N_{CELLS}$ the number of cells in the sample, equal to $4 \times 10^5$

$\% PMN$ the percentage of PMN in the sample

$\% VIAB$ the percentage viable PMN in the sample

The CL of blood PMN was calculated with the same formula as for milk PMN applying the corrections described by Heyneman et al. (1990) and Hoeben et al. (2000a) to correct for interference of eosinophils. CL of PMN isolated from blood and milk of $E. coli$-infected and non-infected quarters was measured at PIH 6, 12, 18, 24, 48, 72, 144, 216 and 312.

The CL kinetics of PMA and latex stimulated and of non-stimulated blood and milk ($E. coli$ infected and non-infected quarters) PMN were evaluated at PIH 0, 12 and 24 and 72 in details.

### Statistical analyses

A mixed model was fitted to the CL data (AUC of 1000 viable PMN / 30 min) including cow as random effect and period (as categorical variable with levels PIH0, PIH6, PIH12, PIH18-24, PIH48-72 and PIH>72), PMN stimulation methods (latex, PMA or non-stimulated) and location of PMN (blood, $E. coli$-infected quarters and non-infected quarters) and their two-way interactions as fixed effects. In order to study the time evolution of CL after infection, CL in each period was compared with CL just before the infection (time 0) in each of the different settings (method by location combinations) at a Bonferroni multiple comparisons adjusted significance level of 0.01 (5 comparisons).

A similar mixed model (but without stimulation method) was fitted to the viability data, and also the time evolution was analyzed in detail as in the previous analysis.

### Results

#### ROS production of milk and blood PMN

During $E. coli$ infection milk PMN CL increased far more rapidly than CL in blood and non-infected quarters PMN (Table 1; Figure 1). Compared to pre-infection values, ROS load of PMA stimulated milk PMN from $E. coli$-infected quarters significantly increased 7.1, 10.5, 9.4 and 2.8-folds at PIH 6, 12, 18-24 and 48-72, respectively; whereas the ROS load for latex stimulation significantly increased 6.8, 9.8, 9.1 and 3-folds (Table 1; Figure 1b). Only a slight non-significant increase was observed in non-infected quarters, from AUC 1044 to roughly 1500 (Table 1; Figure 1c). For blood PMN, ROS load differed significantly from pre-infection values only at PIH 48-72 at which time it roughly doubled (from AUC 3925 to 7555) for PMA and increased 2.8-fold (from AUC 3153 to 8767) following latex stimulation (Table 1; Figure 1a).
Non-stimulated PMN CL paralleled the PMA- and latex- stimulated PMN CL both for blood and milk (infected and non-infected quarters) PMN, but at much lower absolute values. A significant difference with pre-infection values was only observed in the infected quarters at PIH 12 and PIH 18-24 (Table 1; Figure 1c). Both in milk and blood, the pre-infection PMA stimulated CL was higher than latex stimulated CL. Throughout the *E. coli* infection, overall AUC values for CL obtained with different stimulation differed significantly from each other (P<0.001), with AUC equal to 3432 for latex stimulation, 3835 for PMA stimulation and 1574 for non-stimulation. The average differences between latex-stimulated versus PMA-stimulated CL were -402±177 (P=0.024), latex-stimulated versus non-stimulated CL 1858±178 (P<0.001) and non-stimulated versus PMA-stimulated -2260±177 (P<0.001). The average CL differences between blood PMN versus non-infected quarters PMN were 1895±174, blood PMN versus *E. coli*-infected quarters -1073±175, and non-infected quarters’ PMN versus infected ones -2968±176 (P<0.001).

Figure 2 shows CL profiles of PMA stimulated, latex stimulated and non-stimulated blood (Figures 2a; b; c) and milk PMN in infected (Figures 2d; e; f) and non-infected (Figures 2g; h; i) quarters at PIH 0, 12, 24 and 72. The shape differed, somehow, for PMA and latex stimulated PMN CL, especially for PMN from *E. coli* infected quarters. The CL response for *E. coli* infected quarters PMN following PMA stimulation showed a biphasic course with immediate large peak followed by a delayed small peak at PIH 12 and 24. During the same period, maximum CL of milk PMN from non-infected quarters increased slightly. At PIH 72, CL intensity and durability did not alter significantly in PMN of either quarter.

**Viability of milk and blood PMN**

The viability of blood PMN did not change significantly throughout the experiment, and constantly remained high (98±1%). Before *E. coli* challenge, the viability of PMN isolated from milk was 63±2%. The viability of PMN isolated from milk of *E. coli*-infected quarters at PIH 6, 12, 18-24 and 48-72 increased to 86, 93, 89 and 76%, respectively (P<0.001). Pre-infection values were obtained at PIH >72 (Table 2; Figure 3). The viability of PMN isolated from milk of non-infected quarters at PIH 6 increased significantly to 77% (P>0.001) (Table 2; Figure 3). Throughout the *E. coli* infection, average viability values for blood PMN, infected quarters PMN and non-infected quarters were 98, 78 and 68%, and they were significantly different from each other (P<0.001). The overall viability differences between blood PMN versus non-infected PMN was 31±1.5%, blood PMN versus infected quarters was 20±1.6% and non-infected quarters PMN versus infected quarters ones was -11±1.7% (P<0.001).

**Clinical observations, enumeration and differentiation of blood and milk PMN**

Acute mastitis caused by *E. coli* inoculation provoked local as well as systemic effects: inflammation of the *E. coli*-infected quarters with an increase of cfu and SCC, decreased MP, fever and increased TPR (temperature, pulse and respiration). Most clinical signs peaked at PIH 6-12 and completely restored at PIH 72-96 (see e.g., Figure 4). Leukopenia was observed between PIH 12 and 18, regaining pre-infection value at PIH 48 and onwards (Table 3). After decreasing at PIH 6 then increasing at PIH 12, number of circulating PMN was minimal at PIH 18-24, remaining low even at PIH 72...
Number of band cells doubled in the circulation at PIH 6 and slightly higher at PIH 12 then roughly tripled at PIH 18 to 72 and remained substantially high at PIH >72. Metamyelocytes and myelocytes were increased at PIH 6, peaking at PIH 48-72 and remained high at PIH >72.

At PIH 6 to 24, a sharp increase of SCC was observed in E. coli-infected quarters, coinciding with increased cfu (Figure 5). The SCC in control quarters did not change. Before E. coli injection, the percentage of PMN in isolated milk cells was 46±3%. Compared to pre-infection values, the percentage milk PMN almost doubled at PIH 6 to 24 in E. coli-infected quarters, remained a third higher at PIH 48-72, finally regaining the pre-infection value at PIH >72. However, in non-infected quarters the % of PMN did not change during infection (data not shown).

First appearance of E. coli in the milk was seen at PIH 1 and from PIH 3 phagocytosed E. coli were visible (figure 6).

Discussion

Acute mastitis caused by E. coli administration provoked local as well as systemic effects: inflammation of the E. coli infected quarters with an increase of SCC at infection sites, decreased milk production, increased temperature, pulse and respiration and transient leukopenia and neutropenia as seen in previous experiments (Blum et al., 2000; Hoeben et al., 2000a; Mehrzad et al., 2001b). These local and systemic effects change the functional status of PMN in blood and in E. coli infected- and non-infected quarters, but it remains unclear which molecules are involved. For instance, different host-derived cytokines such as tumor necrosis factor-α (TNF-α) (Blum et al., 2000; Hoeben et al., 2000b) interleukin-1 (IL-1), IL-6 (Shuster et al., 1993; Hagiwara et al., 2001) affect PMN function in blood and milk.

This study followed up blood and milk PMN CL and their kinetics during E. coli mastitis, emphasizing both phagocytosis and ROS generation capacity changes. Pathogenesis and outcome of E. coli mastitis are influenced by recruitment of PMN in milk and their bactericidal capacity at the infection site (Kehrli et al., 1989; Kremer et al., 1993; Shuster et al., 1996). After infection, PMN diapedesis in the infected quarters accelerates as SCC (> 95% PMN) increases sharply, which demonstrates the importance of the blood/milk barrier in inflamed quarters affected by bacteria. The increased SCC in milk triggers more PMN recruitment, potentially limiting tissue injury and promoting recovery of severe inflammation (Carey et al., 1997). The activated and activating properties of PMN together with host-pathogen interactions causes systemic effects such as fever and increasing bone marrow output of leukocytes to replenish the circulating pool (Goff et al., 1992). As a result, the transient increase of immature neutrophils in circulation in the present study was expected. The host-derived cytokines also enhance permeability of the blood/milk barrier and exudation of serum albumin in lacteal secretions (Kehrli et al., 1994). Increased permeability of the blood/milk barrier was also indicated by the appearance of clots in milk at PIH 12. Between PIH 6 and 12 a large number of PMN having accumulated in lacteal secretion of inflamed quarters resulted in a substantial increase of the SCC.

Increased milk SCC in E. coli-infected quarters coincided with increased CL and viability of milk PMN as well as a maximal decline of milk production. This is due, in part, to the massive and quick migration of circulating PMN, resulting in milk PMN
viability and CL activity comparable to that of blood PMN. The CL activity of milk PMN exceeded that of blood PMN, suggesting an increased effectiveness of milk PMN upon accumulation in the inflamed quarters following bacterial exposure. During mastitis, milk levels of host-derived cytokines such as TNF-α and IL-6 are higher than blood levels at PIH 4 to 18 (Hoeben et al., 2000b; Hagiwara et al., 2001). Thus, TNF-α and IL-6 might be involved in the observed increase in the milk PMN CL. This observation is in agreement with previous findings on endotoxin mastitis (Mehrzad et al., 2001b) and the results of Watson et al. (1997) who demonstrated that rat PMN isolated from *E. coli* endotoxin-treated bronchoalveolar lavage showed a higher CL activity than circulating PMN.

Blood PMN CL with PMA or latex stimulation or without stimulation did not change substantially during the first day of infection. This was in contrast with Heyneman et al. (1990) and Van Oostveldt et al. (1999) who observed a sudden decrease in PMN ROS generation within day 1 of *E. coli* infection. The most probable reason for this discrepancy is the use of heifers whose blood PMN function is more pronounced as compared to older cows (Mehrzad et al., 2002). The substantial increase of latex and PMA stimulated blood PMN CL at PIH 48 and 72 reveals higher phagocytic and enzymatic activity. This could be due to the production of additional inflammatory mediators, priming the newly released PMN from the bone marrow. It remains unclear which molecules are responsible for an enhanced ROS production and phagocytosis capacity of blood PMN at PIH 48 and onwards in heifers. In blood and non-infected quarters, PMN reacted with latex less than they did with PMA, however, at PIH 18-24 it was reversed. Conversely, in *E. coli*-infected quarters PMN produced more ROS with PMA than latex. The underlying mechanism of these differences remains to be investigated.

More important than blood PMN CL is the increased milk PMN CL. PMN CL of *E. coli*-infected quarters increased much more rapidly than blood PMN CL both for PMA and latex stimulation. This could intensify overall killing capacity and bacterial removal from the infected quarters. The differences in kinetics of PMA and latex stimulated CL of PMN from *E. coli* infected quarters were apparent. The steady slope in PMA stimulated CL and highest intensity and durability at PIH 12 could result from activation of both extra- and intra-cellular enzymatic activity. This exhibited more intracellular bactericidal efficiency at PIH 6, 12 and 24, resulting in declined cfu at PIH 24. While many intracellular bactericidal mechanisms of PMN have been described (Reeves et al., 2002), the crucial role of ROS on bactericidal mechanisms is nevertheless apparent (Allen et al., 1972; Grebner et al., 1977), particularly on Gram-negative bacteria (Burvenich et al., 1994).

The CL kinetics of PMA and/or latex-induced luminol-dependent CL also reveals some details of intra-and-extracellular ROS generation (Rosen and Klebanoff, 1976; DeChatelet et al., 1982). Clearly, after 3-4 minutes of luminol dependent CL kinetics, ROS production results mainly from intracellular events (DeChatelet et al., 1982; Edwards et al., 1986; Faulkner and Fridovich, 1993). Further, luminol-dependent CL requires H₂O₂ (Lind et al., 1983; Edwards et al., 1986; Faulkner and Fridovich, 1993). Indisputably, intracellular H₂O₂ production is maximal in PMN from *E. coli*-infected quarters at PIH 6 and 12 to 18. The H₂O₂ is the precursor of variety of subsequent powerful ROS such as HOCL (Chapman et al., 2002). The shape of latex stimulated CL of PMN of *E. coli* infected quarters emphasizes increased factors related to
phagocytosis activity, boosting bacterial exposure to intracellular ROS during early phase of infection. Consequently, the phagolysosome of PMN uses their intracellular components e.g. MPO- H₂O₂-Cl system to chlorinate bacterial proteins (Rosen et al., 2002), thereby eliminating phagocytosed *E. coli*. Certainly, the particular CL shape reveals the quality of milk PMN, because the milk PMN had maximal viability as well.

Non-stimulated milk PMN of infected quarters also showed substantially higher ROS production than those of blood and non-infected quarters. This might be due to increased production of host and/or pathogen-derived cytokines in infected quarters (Hoeben et al., 2000b; Blum et al., 2000; Hagiwara et al., 2001), thus increasing milk PMN ROS production capacity. The slight increase of CL intensity and durability in PMN of non-infected quarters unequivocally shows a systemic effect of *E. coli* infection in heifers.

The modulation of bovine PMN viability in milk by *E. coli* mastitis is related to the kinetics of PMN diapedesis through the blood/milk barrier. Still, the precise mechanism of increased milk PMN viability during *E. coli* mastitis is unclear. It might also be related to the anti-apoptotic effect of cytokines like TNF-α, IL-1 and IL-6 or bacterial LPS (Colotta et al., 1992; Lee et al., 1993; Sweeney et al., 1998). Milk concentration of all of these host- and pathogen-derived cytokines is extremely high throughout day 1 of *E. coli* mastitis (Shuster et al., 1993; Blum et al., 2000; Hoeben et al., 2000b; Hagiwara et al., 2001). Additionally, contribution of neutrophil NADPH-oxidase activity to PMN survival and cytosolic pH homeostasis is pivotal (Mayer et al., 1989; Jankowski et al., 2002), possibly resulting in prevention of fast PMN cytosolic acidosis and necrosis. Investigating the milk PMN cytosolic pH during *E. coli* infection could provide fundamental evidence on milk PMN viability alterations. There could also be other systemic and local factors, such as recruitment of younger PMN particularly in the *E. coli* infected quarters resulting in increased viability. Furthermore, the fast diapedesis may explain the delayed apoptosis and increased viability of PMN at the site of infection on the basis of the influx of younger PMN (Ferrante, 1992; Lee et al., 1993; Watson et al., 1997; Mehrzad et al., 2001b). The contribution of the blood-milk barrier (Van Oostveldt et al., 2002) and mammary gland injury (Sladek et al., 2001) in the modulation of PMN apoptosis has been recently reported. In non-infected quarters, surprisingly, an increase of milk PMN viability, though less pronounced, was observed as well. This emphasizes the systemic effect of *E. coli* mastitis on milk PMN function. The mastitis-related milk PMN viability modulation, which might prevent inappropriate or prolonged inflammation, is a very complex and interesting issue and warrants further investigation. Whether increased milk PMN viability during the early hours of infection enhances mammary gland immunity against invading pathogens is as yet unknown. Nevertheless, in *E. coli*-challenged quarters PMN viability and CL were inextricably interrelated, offering a potentially more effective and stronger first line defense mechanism for the mammary gland during infection. The early phase of increased milk PMN CL and viability is critical in the termination of inflammation.

It appeared heifers reacted against *E. coli* infection efficiently and local and systemic effects of *E. coli* mastitis were transient. Fast diapedesis dramatically influences milk PMN CL and viability. During the early phase of mastitis blood PMN is not activated substantially; however, when PMN migrate in infected quarters their functionality
enhances sharply. This might be due to the kinetics of PMN diapedesis during infection, resulting in a significantly higher PMN CL and viability in \textit{E. coli} infected quarters than blood and non-infected quarters.

\textbf{Acknowledgements}

Ministry of Agriculture and Small Enterprises of Belgium (S/6014 and S/5871), Ministry of Science, Research and Technology of Iran and the Research Fund of Ghent University (BOF-project; Grant 011D4001) provided financial support of the study. We also thank K. Demeyere and E. Vander Elstraeten for technical assistance.

\textbf{References}

Table 1: PMA, latex-and non-stimulated chemiluminescence of blood and milk (infected and control quarters) PMN prior to and during experimentally induced *E. coli* mastitis. Values are means ± SEM of 15 cows. Each timepoint is compared with time 0 (before challenge) with an asterisk denoting a significant difference at comparisonwise error rate equal to 0.01.

<table>
<thead>
<tr>
<th>Source of PMN</th>
<th>Stimulator</th>
<th>PIH</th>
<th>Blood</th>
<th>Infected quarters</th>
<th>Control quarters</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0</td>
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<td>1086±512</td>
<td>1044±513</td>
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<tr>
<td></td>
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<td>3763±696</td>
<td>7710±691*</td>
<td>1646±693</td>
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<tr>
<td></td>
<td>12</td>
<td>2739±691</td>
<td>11395±697*</td>
<td>1583±685</td>
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<tr>
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<td>18-24</td>
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<td>10237±512*</td>
<td>1634±512</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48-72</td>
<td>7555±514*</td>
<td>3003±512*</td>
<td>1553±513</td>
<td></td>
</tr>
<tr>
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<td>1800±431</td>
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<tr>
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<td>Non-stimulated</td>
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<td>6</td>
<td>98.7±3.2</td>
<td>85.5±3.7*</td>
<td>77.0±3.2*</td>
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</tr>
<tr>
<td></td>
<td>12</td>
<td>98.0±3.2</td>
<td>92.8±3.8*</td>
<td>66.8±3.3</td>
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<td></td>
<td>18-24</td>
<td>98.4±2.5</td>
<td>88.7±2.8*</td>
<td>70.9±2.4</td>
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</tr>
<tr>
<td></td>
<td>48-72</td>
<td>98.0±2.5</td>
<td>75.3±2.7*</td>
<td>63.6±2.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;72</td>
<td>98.4±2.1</td>
<td>62.4±2.2</td>
<td>59.7±2.4</td>
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Table 2: Viability fluctuations of blood and milk (infected and control quarters) PMN during experimentally induced *E. coli* mastitis. Values are means ± SEM of 15 cows. Each timepoint is compared with the time 0 (before challenge) with an asterisk denoting a significant difference at comparisonwise error rate equals to 0.01.
Table 3: Parameters of blood cells measured during experimentally induced *E. coli* mastitis in dairy heifers. Values are means ± SEM of 15 cows.

<table>
<thead>
<tr>
<th>PIH</th>
<th>Parameters</th>
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<th>48-72</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>WBC/µl</td>
<td>PMN/µl</td>
<td>Band cell/µl</td>
<td>Meta +Myelo/µl</td>
<td>Lymphocyte/µl</td>
<td>Eosinophil/µl</td>
<td>Monocyte/µl</td>
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<tr>
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<td>4370±325</td>
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<td>54±17</td>
</tr>
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<td>12</td>
<td>3426±736</td>
<td>365±86</td>
<td>173±51</td>
<td>813±216</td>
<td>1883±444</td>
<td>141±62</td>
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<tr>
<td>18-24</td>
<td>6805±621</td>
<td>1367±249</td>
<td>689±117</td>
<td>1999±247</td>
<td>2567±249</td>
<td>179±82</td>
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<td>48-72</td>
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<td>3165±238</td>
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<td>1852±155</td>
<td>4826±224</td>
<td>686±67</td>
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Figure 1. PMA (dotted lines), latex (solid lines) and non-stimulated (dashed lines) chemiluminescence of PMN isolated from blood (a) *E. coli*-infected (b) and non-infected (c) quarters of cows during experimentally induced *E. coli* mastitis. Values are the mean of 15 cows. Data are expressed as the area under the curve (AUC) of continuously ROS generation of $10^3$ viable PMN for 30 minutes.
E. coli mastitis and its effect on PMN respiratory burst activity and viability

Figure 2. Chemiluminescence profile of PMA (upper row) and latex (middle row) stimulated and of non-stimulated (bottom row) PMN from blood and milk, infected and non-infected, quarters at PIH 0, 12, 24 and 72 of experimentally induced E. coli mastitis. The generation of CL was monitored continuously for 30 min after addition of 100 ng/ml PMA and/or 500 latex beads particles/PMN or addition of none of them and then 0.3 mM luminol to the 4. 10^5 isolated PMN suspension in 200μl. The curves are average of 5 cows.
Figure 3. Viability of milk PMN isolated from \textit{E. coli}-infected (solid line) and non-infected (dotted line) quarters and of blood (dashed line) of cows during experimentally induced \textit{E. coli} mastitis. Values are the mean of 15 cows.
Figure 4. Daily milk production (liters/udder halve) from the *E. coli*-infected (closed bars) and non-infected quarters (open bars) of cows during *E. coli* mastitis. Values are the means ± SEM of 15 cows. Time 0 = day of *E. coli* inoculation.
Figure 5. Multiplication rate of *E. coli* (solid line) in infected mammary glands and pattern of leukocyte influx (dashed line) into milk during experimentally induced *E. coli* mastitis.
Figure 6. Light microscopic picture of isolated PMN from *E. coli* infected quarters at PIH 6 (a), 12 (b), 18 (c), 24 (d) 48 (e) and 72 (f). *E. coli* is phagocytosed by milk PMN 6 hours after the bacterial suspension was inoculated into the mammary gland. The presence of immature neutrophils is visible.
Part 4

Study on susceptibility for
*E. coli* mastitis
Chapter 8

Chemiluminescence of blood and milk neutrophils as a predictive factor for severity of *Escherichia coli* mastitis in dairy cows
Abstract

The polymorphonuclear leukocytes (PMN) function changes during mastitis. To investigate the contribution of blood and milk PMN function to the severity of *E. coli* mastitis, chemiluminescence (CL) of PMN and their efficiency to destroy coliform bacteria in the mammary gland were examined following induction of *E. coli* mastitis in early lactating cows. Cows were classified in moderate (M) and severe (S) responders according to milk production loss (MPL) in the non-challenged udder halves at post-infection hour (PIH) 48. Pre-infection PMN CL and CL and colony-forming units (cfu) during infection differed in M and S responders. In M responders, the pre-infection blood and milk PMN CL was ~2-fold higher than that of S responders. The probability of severe response increased with decreasing pre-infection CL mainly in milk PMN but also in blood PMN. During the early phase of infection, blood and milk PMN CL was consistently higher in M cows and milk PMN CL increased immediately after infection in M cows. At PIH >48 milk PMN CL in S cows superseded that of M cows. The SCC in M cows increased faster than cfu after infection, whereas in S cows the reverse was observed. CL kinetics of PMA and latex stimulated blood and milk PMN before and in the early stage of *E. coli* infection in S cows suggests intracellular CL impairment, further explaining bactericidal inefficiency in S responders. Blood PMN differentiation was also different in M and S responders. High blood and milk PMN pre-infection CL and the immediate increase of milk PMN CL and SCC after infection prevents uncontrolled bacterial growth, thus facilitating recovery of coliform mastitis in M responders. Our findings suggest that low blood and milk PMN CL are risk factor for severe clinical coliform mastitis in dairy cows.
Introduction

It has been shown that ingestion of bacteria by polymorphonuclear leukocytes (PMN) triggers many bactericidal mechanisms (Reeves et al., 2002) including marked increase in cyanide-insensitive oxygen consumption and the generation of reactive oxygen species (ROS) such as $\text{O}_2^-$, $\text{H}_2\text{O}_2$, $\text{OH}^-$, $1\text{O}_2$ and HOCl (Bellavite, 1988; Babior, 1984). These ROS are pivotal for killing engulfed bacteria (Thomas et al., 1982; Weber et al., 1983; Dulin et al., 1988; Kehrli et al., 1989; Mehrzad et al., 2001a). The most immediate response to acute mastitis is massive recruitment of PMN from the circulation into the mammary gland. Although several antimicrobial systems exist in the mammary gland (Paape et al., 1979; 1996; Kehrli et al., 2001), it is generally accepted that the presence of PMN in milk provides a central natural defense for the gland (Burvenich et al., 1994).

Despite the importance of PMN function in E. coli mastitis, little is known about the role of PMN ROS in bacterial destruction during E. coli mastitis. PMN ROS can be measured by the phagocytosis-induced and/or non-induced chemiluminescence (CL) assay, which is the most widely used technique to quantify PMN ROS load (Allen et al., 1972; Weber et al., 1983; Piccinini et al., 1999; Mehrzad et al., 2001a).

The different CL responsiveness of blood and milk PMN to soluble/particle stimuli during pathological conditions could be due to different factors such as differences in protein kinase C, NADPH-oxidase and myeloperoxidase (MPO) activities (Webb et al., 1974; Babior, 1984). As these enzyme activities reflect intracellular and extracellular reactions, observed differences might offer some explanation about the differences in the cow’s response against invading pathogens in the mammary gland. Under both clinical and experimental conditions, mastitis cows show a large variability in illness and a wide range of pathological responses (Heyneman et al., 1990; Vandeputte-Van Messom et al., 1993; van Werven et al., 1997). Previous studies conducted during physiological and pathological conditions of the mammary gland have also highlighted the considerable variations in blood and milk PMN CL (Mehrzad et al., 2001a; b; Mehrzad et al., 2002). Despite the observed hematopoeigenesis and PMN recruitment into milk during the course of inflammation, it is unknown whether the recruited milk PMN makes a difference in response to invading pathogens to the mammary glands.

This study was conducted to investigate: 1) the relationship between pre-infection blood and milk PMN CL and severity of inflammation following inoculation of E. coli in the udder; and 2) the capacity of blood and milk PMN CL to eliminate phagocytosed bacteria during E. coli infection.

Materials and methods

Animals

All Holstein-Friesian cows were in their 215±6 days of first pregnancy (2.2±0.3 yr) on arrival at the dairy farm (CDFO- Commercial Dairy Farm Oudenaarde, Belgium). The animals were on a system of zero-grazing from arrival till the end of the experiment. They were put in an individual stall and were fed with a special ration for pregnancy and lactation and always had free access to water and hay. After gestation, clinically healthy cows (free from typical periparturient diseases before and after calving) were selected (n=20) on the basis of 2 consecutive bacteriologically negative milk samples and a milk somatic cell count (SCC) of $< 2 \times 10^5$ / ml milk per individual quarter. One
week before infection, the animals were fed a daily ration of approximately 8 kg of concentrate and had free access to water and hay. They were milked twice daily at 0800 and 1800 with a 4-quarters milking machine. Intramammary (i.mam) *E. coli* were inoculated in animals at 19±5 days after parturition.

**Bacterial challenge**

*Escherichia coli* (the P4:032 strain) isolated from a clinical case of mastitis was used. This strain has been frequently used to induce bovine mastitis by several researchers. The stock of *E. coli* was maintained in lyophilized medium at −20 °C until use. Cultures were frequently observed for viability and purity. Before infection, the bacteria were subcultured in brain-heart infusion broth (CM225; Oxoid, Nepean, ON, USA) at 37 °C. The bacterial suspension was washed 3 times with pyrogen free saline solution (0.9%) and resuspended in the solution. Bacterial counting was performed using the plate count method. Before *E. coli* injection the teat ends were disinfected with ethanol (70%) mixed with 0.5% chlorohexidine. *E. coli* mastitis was induced – after the morning milking - into the left front and rear quarters by a single i.mam injection of 10 ml of 10⁴ cfu *E. coli* P4:032 solution per quarter using a sterile teat cannula (7cm; Me. Ve. Mat., Deinze, Belgium). After injection, each quarter was massaged for 30 sec to distribute the bacterial solution in the gland.

**Milk and blood sampling and clinical symptoms**

Individual quarter milk samples were aseptically collected for determination of cfu (10 ml), somatic cell count (SCC) (50 ml) and isolation (200 ml) at 24 h before, immediately before and at 6, 12, 24, 48, 72, 144, 216 and 312 hours following *E. coli* injection. For bacteriological examination of milk to cfu enumeration, 0.5 ml of quarter milk was serially diluted in a pyrogen free saline solution (0.9%) and 0.01 ml of samples of different dilution were streaked in duplicates on Columbia Sheep Blood Agar (Biokar Diagnostics, Beauvois, France) plates using an inoculation loop. The plates were incubated for 24 h at 37 °C. Peripheral blood (80 ml) was collected aseptically from each cow by venipuncture from the external jugular vein into evacuated tubes (Laboratory EGA, F-28210 Nogent-le Roi, France) containing 125 i.u. heparin as anticoagulant. The blood sampling was carried out subsequently after milk sampling at 24 h before, immediately before and at 6, 12, 24, 48, 72, 144, 216 and 312 hours following *E. coli* injection. Measurements of rectal temperature (RT), heart rate (HR), rumen motility and clinical examination of the mammary gland were performed at the time of blood and milk sampling. Evening and morning milk were pooled to obtain daily milk production (MP). To assess the severity of *E. coli* induced mastitis, the MP loss (MPL) of non-infected udder halves at post-infection hours (PIH) 48 of mastitis induction compared to the pre-infection MP was derived (Shuster et al., 1996). Cows were divided into two different severity groups: moderate with MPL<50% (M; n=15) and severe (S; n=5) with MPL≥50%.

**Blood and milk parameters before *E. coli* challenge**

Blood and milk was collected in healthy cows for PMN isolation before *E. coli* challenge and blood and milk PMN ROS production capacity was determined. The MPL of uninfected quarters and cfu of infected quarters were measured at PIH 48 and 6, respectively.
Blood and milk parameters after *E. coli* challenge
The respiratory burst of blood and milk PMN was followed up for several days after *E. coli* infection. Particularly the PMA-and latex stimulated PMN CL kinetics, cfu, SCC and differentiation of circulating leukocytes was performed throughout the study.

Blood and milk PMN preparation, enumeration and differentiation
All materials and reagents used for the isolation of blood and milk PMN were sterile. Isolation of PMN from peripheral blood was performed using hypotonic lysis of erythrocytes (Carlson and Kaneko, 1973). The isolation procedure of PMN from blood yielded > 98% of granulocytes (PMN + eosinophils) with predominantly PMN (> 87%) and a viability of > 98%. After counting the cells using an electronic programable particle counter (Coulter counter Z2, Coulter Electronics Ltd., Luton., UK) and determining the viability and percentage of PMN, the cell suspension was adjusted to a concentration of 5.10⁶ cells/ml in Dulbecco's phosphate buffered saline (DPBS; Gibco BRL, Life Technologies Inc., MD, USA) supplemented with gelatin (0.5 mg/ml; Merck, Darmstadt, Germany). Individual quarter milk samples were used for subsequent PMN isolation, as described previously (Mehrzad et al., 2001b). Briefly, pooled milk of the 2 *E. coli*-infected quarters of each cow was filtered separately through a nylon filter (40µm pore size) and diluted to 60% v/v with cold DPBS. Isolation of PMN from milk was performed using three centrifugation steps as previously described (Mehrzad et al., 2001a,b). The isolation procedure yielded 65-98% PMN with viability (determined in duplicate by means of flow cytometry (FACSScan, Becton Dickinson Immunocytometry Systems, San José, CA, USA), using propidium iodide exclusion) 70-98% throughout the experiment. The total number of leukocytes and isolated blood and milk cells were determined using an electronic particle counter (Mehrzad et al., 2001a). The total number of different circulating leukocytes was determined using smear preparation of blood sample (Mehrzad et al., 2001a). Differential cell counts and staining procedure were performed on whole blood similar to the isolates on eosin-Giemsa-stained smears, using light microscopy with the identification based on morphological characteristics as described by Hayhoe and Flemans (1969); McDonald and Anderson (1981) and Mehrzad et al. (2001a). To quantify percentages of each cell type in the samples, PMN (mature and immature), monocytes/macrophages, lymphocytes, eosinophils and epithelial cells (only in milk) were identified on 200 cells per slide and expressed as % of particular cells in respective samples.

Chemiluminescence assay
Luminol-enhanced PMA (phorbol 12-myristate, 13-acetate)-and-latex beads (polystyrene 0.76 µm diameter, 4.10¹¹ particles / ml; Sigma)-stimulated cellular CL was used to measure the CL activity of PMN isolated from blood and milk of *E. coli*-infected quarters. CL was measured in duplicate during 30 min at 37 °C with a microtiterplate luminometer (type LB96P; EG&G Berthold, D-75312 Bad Wildbad, Germany). PMA-stimulated CL was measured immediately after addition of 100 ng/ml PMA and 0.3 mM luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione, Sigma) to 2.10⁶ cells/ml in a total volume of 200 µl per well. Similar concentrations of luminol and cells per well were used for latex beads (final concentration of 500 particles/PMN) stimulated CL. Stock solutions of PMA and luminol were prepared in dimethyl sulphoxide (DMSO) (Sigma) and always stored at –20 °C. The area under the curve (AUC) was calculated for the registered impulse rates (relative light unit
(RLU)/s) over the whole measurement period of 30 minutes. The CL response was expressed per $10^3$ viable PMN in each isolated cell sample. The contribution of milk macrophages to luminol-dependent CL is negligible (Mehrzad et al., 2001a), the CL response was expressed per $10^3$ viable PMN. For milk PMN CL assay the formula, $\text{CL}_{\text{PMN}} = 10^3 \times \text{Cl}_{\text{isolated cells}} / (4.10^5 \times \% \text{PMN} \times \% \text{V})$, was used to perform the corrections, where Cl = mean RLU (relative light unit)/s, $4.10^5 = \text{total number of cells per well}$, $\% \text{PMN} = \text{total percentage of PMN in isolated cells}$, $\% \text{V} = \text{percentage of viable PMN}$. The CL of blood PMN was calculated with the same formula as for milk PMN applying the corrections described by Heyneman et al. (1990) and Hoeben et al. (2000) for interference of eosinophils.

The CL kinetics of blood and milk PMN stimulated by PMA and latex was performed prior to and during the course of *E. coli* challenge in all individual cows throughout the study.

**Statistical analyses**

The relationship between CL (AUC) immediately before challenge and reduction of milk production 48 hours after challenge was first studied by a linear regression model and the null hypothesis of the slope being equal to 0 was tested. Alternatively, logistic regression analysis was performed to investigate whether CL (AUC) immediately before challenge can predict whether a cow will be a severe responder (reduction of milk production 48 hours after challenge $>50\%$) or not. Again the null hypothesis of the slope in the logistic regression model being equal to 0 was tested. Furthermore, cfu at 6 hours was linearly regressed on the CL (AUC) immediately before challenge and the null hypothesis of the slope being equal to 0 was tested.

The differences in CL after challenge between moderate and severe responders were analysed by a mixed model with cow as random effect and time as categorical variable with 5 levels (0, 6, 12, 18-24 and $\geq 48$ hours) for each of the two locations (blood or milk) and for each of the two methods of stimulation (latex or PMA). Such analyses were done for AUC, $\text{RLU}_{\text{max}}$ and $T_{\text{max}}$ as dependent variables to study different aspects of the CL process. As analyses are done at 5 different timepoints, each comparison between moderate and severe responders is performed at the 0.01 significance level in order to ensure an overall size equal to 0.05 (Bonferroni’s multiple comparisons technique).

**Results**

**Pre-infection PMN CL in blood and milk in relation to severity of mastitis**

The level of pre-infection CL (AUC) in milk PMN has a significant influence on the severity of mastitis. Both MPL and the probability of severe response decrease significantly with increasing values of pre-infection CL (AUC) in milk PMN with both PMA and latex stimulation (Figures 1.b and 1.d, Table 1). Although the same trend was observed in blood PMN, a significant result was only obtained for the effect of PMA stimulated pre-infection CL on the probability of severe response ($P=0.038$), and the linear effect is generally smaller in blood PMN.

Pre-infection CL for both milk and blood PMN significantly influences another parameter of mastitis severity, cfu at PIH 6, but again the relationship was far stronger in milk PMN (Table 2).

Figure 2 shows the kinetics of blood and milk PMN CL immediately before infection after stimulation with PMA and latex beads of the individual M and S cows prior to infection. Both for blood and for milk PMN stimulated with either latex or PMA the
PMN chemiluminescence as a predictive factor for severity of Escherichia coli mastitis

lowest CL values were observed in S cows. In the presence of PMA, pre-infection blood PMN RLU_{max} in M cows was never below 2000 RLU/s, whereas in S cows the RLU_{max} never reached 2000 RLU/s. In milk, though lower than blood, the average RLU_{max} in M cows was above 200 RLU/s, ~ 2-fold higher than that of S cows. Both for milk and for blood PMN CL induced with PMA T_{max} was always higher in M cows (Figures 2.c and 2.d). In the presence of latex, though slightly lower than PMA, similar patterns of blood and particularly milk PMN CL for RLU_{max} and T_{max} were observed (Figures 2.a and 2.b). Pre-infection PMN AUC of M cows was ~ 2-fold higher than those of S cows in each particular combination of blood and milk PMN with latex and PMA. Furthermore, the PMA and latex stimulated blood PMN CL in M responders increased faster, remained substantially higher for a longer time and decreased more slowly than in S responders (Figures 2.a and 2.c). This pattern was similar to that of milk PMN (Figures 2.b and 2.d).

Blood and milk differentiation between M and S responders and clinical symptoms

Although no significant differences on pre-infection WBC and circulating PMN were observed between the moderate and severe groups, leukopenia and neutropenia was far more pronounced in S cows, with neutropenia existing even at PIH > 48 in S cows (Table 3). The overall pre-infection SCC was the same for M and S cows. The SCC in M cows, however, increased faster (maximal at PIH 6). In S cows the maximal SCC appeared at PIH 18-24 (Figure 3; Table 3). Intramammary E. coli infection induced an increase of RT and HR that peaked at PIH 6 to 12, as well as swelling and pain of the infected quarters, appearance of flecks and milk leakage in infected quarters observed at PIH 6 to 12 (data not shown) for both groups. All clinical symptoms disappeared in M cows within PIH 24, but lasted longer in S cows. Clinical signs were more pronounced in S cows and even at PIH >72 infected quarters caused pain and had abnormal secretion.

Effect of E. coli mastitis on blood and milk PMN CL kinetics in M and S responders

Figure 4 shows blood and milk PMN CL kinetics after stimulation with PMA and latex beads during mastitis in M and S responders. In latex and PMA activated blood PMN, consistently higher ROS capacity in M cows was observed throughout the infection. Moreover, a quicker shift in activity to higher AUC and RLU_{max} and a tendency from left to the right for latex ingestion in M cows are remarkable (Table 4 and Figure 4; a.1, a.2, a.3, a.4; c.1, c.2). The T_{max} at PIH 18-24 for latex stimulated blood PMN was substantially lower in S cows (Table 4).

Figure 4 and Table 4 also show the PMA and latex activated CL kinetics of milk PMN harvested from E. coli infected quarters during infection. Compared to pre-infection PMN CL shapes, large disparities between M and S groups during E. coli infection were observed. For latex stimulated milk PMN CL kinetics, in addition to an overall higher AUC and T_{max}, the average RLU_{max} in the M group at PIH 6 and 12 was ~ 2000, whereas it was bellow 650 in the S cows (Table 4; Figure 4, 2.b and 3.b). There was also a prolonged shift of curve from left to the right for latex ingestion in M cows at PIH 6, 12, 18 and 24 (Table 4; Figure 4, 3.b, 4.b and 5.b). At PIH 48 and 72 RLU_{max} and AUC of latex stimulated milk PMN of S cows revealed a reversed response compared to M cows (Table 4; Figure 4, 6.b and 7.b). For PMA stimulated milk PMN CL kinetics, a significantly higher T_{max} was only observed at PIH 18-24. Overall, higher AUC appeared in the M cows and the average RLU_{max} in M cows at
PIH 6 and 12 was 2-fold higher than in S cows (Table 4; Figure 4, 2.d and 3.d). There was also a biphasic pattern in PMA-stimulated PMN CL at PIH 6, 12, 18 and 24 of M cows (Figure 4, 2.d, 3.d and 4.d). While at PIH 6 and 12 the first peak is bigger, at PIH 18 and 24 the second peak is higher (Figure 4, 2.d, 3.d and 4.d). Surprisingly, at PIH 48 and 72 RLU$_{\text{max}}$ and AUC of PMA stimulated milk PMN of S cows revealed reversed response compared to M cows (Table 4; Figure 4, 6.d and 7.d).

**Discussion**

Blood and milk PMN CL in M and S responders were already different before infection and changed in a different way during *E. coli* mastitis. Also the speed of PMN mobilization was different between the two groups. Our findings about blood PMN CL are consistent with previous studies (Heyneman et al., 1990; Vandeputte-Van Messom et al., 1993). The assessment of milk PMN CL before and during *E. coli* infection highlighted the important influence of milk PMN CL on severity of *E. coli* mastitis in dairy cows.

Recruitment of PMN with high bactericidal capacity into the udder at the right time is essential in the prevention of severe *E. coli* mastitis (Hill et al., 1979; 1981; Rainard, 1995). Having already recruited PMN in milk with higher CL, the M cows cleared the *E. coli* bacteria faster than the S cows. This reveals the crucial role of PMN in the prevention of further adverse inflammatory effects on mammary tissues. Furthermore, uncontrolled ROS generation at the wrong time is harmful for many cell systems e.g. T cell hyporesponsiveness and lymphocyte proliferation inhibition caused by ROS (Nonnecke and Harp, 1988; Cemerski et al., 2002). ROS also enhances natural killer cell and T cell activity (Suthanthiran et al., 1984; Cemerski et al., 2002), indicating that PMN ROS at the right time may not only damage the cells and tissues but may also facilitate recovery of *E. coli* mastitis.

We found that the extent of MPL (an index for severity) depends, in part, on the pre-infection blood and milk PMN CL; it was lower at higher PMN CL. This relationship was most pronounced in milk PMN CL. A rapid bacterial clearance appeared within PIH 18 in M cows which could largely be associated with a high pre-infection CL and rapid influx of PMN at PIH < 6. Therefore, the impact of blood and milk PMN CL on severity was crucial. For example, every unit increase in pre-infection blood and milk PMN CL (AUC) resulted in roughly 5 and 20 ml gain in MPL at PIH 48 respectively, which coincides with a decrease of 0.3 and 0.5% in the probability to develop severe *E. coli* mastitis. This is consistent with the finding of Zecconi et al. (1994) who observed that the probability of developing clinical mastitis in cows with lower milk PMN CL was much higher than those with higher milk PMN CL. The faster increase in SCC in the M cows might also have contributed towards the elimination of *E. coli* bacteria.

Equally important, the milk PMN CL in M cows peaked at PIH 6 and 12, whereas in S cows it occurred much later at PIH 24 resulting in higher cfu in the gland of S cows at each moment in time. As seen in the current study, therefore, the initial bacterial growth is critical to produce further inflammation. The second milk PMN CL peak at PIH 72, at which all pathological and tissue damage had already happened, in S cows is somewhat counterproductive. In contrast, increased milk PMN CL in M cows lasted until PIH 24.
The blood PMN response to PMA and/or latex beads did not decline substantially during the first day of infection in M cows, whereas in S cows this decline was substantial, and also a decline in hematopoeigenesis was observed. This was in accordance with previous findings (Heyneman et al., 1990; Vandeputte-Van Messom et al., 1993); but in our study the decline was faster probably due to the fact that heifers react faster than pluriparous cows (Mehrzad et al., 2002). Latex- and PMA-stimulated blood PMN CL in M responders revealed no substantial changes in the first 24 hours after infection in agreement with Heyneman et al. (1990), who used zymozan and PMA for PMN stimulation. Latex in our study was uncoated, equally implying to non-specific phagocytosis. The highest affinity of blood PMN to uptake latex beads in M responders, as seen in CL kinetics, could partly give an idea about activity of contractile proteins e.g. actin filaments and myosin; they are crucial during phagocytosis (Cox et al., 2002). For example, microtubules in PMN play a central role in membrane invaginations, phagosome formation and internalization of the phagosome during phagocytosis (Hoffstein et al., 1977). It is likely that latex beads are phagocytosed incompletely and thus remain peripheral in PMN of S cows. For the principal mechanism of engulfing E. coli by post-diaptetic PMN in milk, rearrangement of cytoskeleton and pseudopod extension would be pivotal. It remains to be investigated whether cytoskeleton, pseudopod extension and molecular mechanisms in PMN are different in M and S cows.

In contrast to M responders, neither CL nor SCC seemed sufficiently high to decrease cfu in S responders. What makes the M responders more resistant against E. coli mastitis is not merely higher PMN CL but also different PMN CL kinetics before and during E. coli infection play a role. To our knowledge, little is known about the kinetics of either milk or blood PMN CL or their links to the severity during E. coli mastitis. The CL kinetics of either latex or PMA stimulated blood and milk PMN during E. coli infection revealed important differences in M and S responders: RLU_{max} in blood PMN was always remarkably higher in M cows, and also for milk PMN (except at PIH 48 and 72). Thus, the blood PMN and the newly migrated PMN in milk are more efficient in M responders. The higher probability of developing clinical mastitis in S cows could result, at least in part, from lower milk PMN CL intensity before infection and at PIH 6 and 12.

Higher intensity and durability of blood PMN CL prior to and during E. coli infection in M cows reveals PMN’s higher oxygen-dependent intracellular bactericidal capacity. The substantial decrease of blood PMN CL intensity in S cows at PIH 18-24 might be due to inflammation (Mehrzad et al., 2001b). The observation of early strong local response in M cows and of late local but early systemic response in S cows was, in part, responsible for most of the disparities between M and S groups. Other PMNs (e.g. immature neutrophils) were observed in blood (both isolated and whole blood) with a higher frequency in S cows at PIH 18-24. This could have further lowered blood PMN CL in S responders as they produce less ROS than mature neutrophils (Glasser et al., 1987). On the other hand, at PIH 6 the amount of mature neutrophils in M cows was lower with higher CL activity, suggesting that overall oxidation-reduction reactions in mature blood neutrophils of M cows are even higher.

The kinetics of milk PMN CL also revealed smaller intracellular bactericidal efficiency in S cows during pre-infection and at PIH 6, 12 and 24. Although several intracellular bactericidal mechanisms of PMN have been described elsewhere (Reeves
et al., 2002), the central role of ROS on bactericidal mechanisms is nevertheless indisputable (Allen et al., 1972; Grebner et al., 1977), especially for Gram-negatives (Burvenich et al., 1994). Moreover, the CL kinetics of PMA and/or latex-induced luminol-dependent CL reveal some details on the location of the ROS that are produced intra- and extra-cellularly (Rosen and Klebanoff, 1976; DeChatelet et al., 1982). In luminol dependent CL kinetics, ROS production after 3-4 minutes results mainly from intracellular events (DeChatelet et al., 1982; Edwards et al., 1986; Faulkner and Fridovich, 1993). As the luminol-dependent CL requires hydrogen peroxide (Lind et al., 1983; Edwards et al., 1986; Faulkner and Fridovich, 1993), it is highly likely that the intracellular hydrogen peroxide production is higher in PMN from M responders than those from S responders. Subsequently, impairment of intracellular reactions of MPO–H2O2 halide system is more pronounced in S cows, yielding less HOCl. The decreased intracellular events of ROS production could be the main cause of lower milk PMN CL in S responders at PIH 6, 12 and 18, resulting in much higher cfu in milk of S responders after bacterial infection. Since HOCl is major intracellular PMN ROS during phagocytosis and respiratory burst activity (Chapman et al., 2002) and the evidence of milk PMN CL kinetics before and at the early phase of bacterial infection, lower milk PMN HOCl production, in part, would have caused the delayed recovery in S cows. Further studies are in progress to explain the impaired milk PMN bactericidal efficiency at the early phase of bacterial growth in S responders.

Latex- and PMA-stimulated milk PMN CL kinetics in S cows gave neither high AUC nor high RLU as compared to M cows. During the late stage of infection, at PIH 48 and later, however, the AUC and RLU values were higher in the S group. This reaction could be unsuitable for the host because ROS could be produced extracellularly. The low T_{max} is counterproductive, resulting in tissue damage. Overall CL kinetics of PMA and latex stimulated CL both for blood and milk during early phase of inflammation revealed that extracellular CL is almost similar between S and M responders but intracellular CL is impaired in S responders.

In brief, high blood and milk PMN CL at the start and in the early phase of the infection is crucial for removing pathogens from the infection site, thus alleviating severity of *E. coli* mastitis. Increased milk PMN CL and SCC and decreased cfu coincided in M cows, revealing that M cows are far better equipped in fighting off the *E. coli* bacteria. Inflammation reactions, bactericidal activity and tissue damage is much better balanced in M than in S responders.

**Acknowledgements**

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**References**

PMN chemiluminescence as a predictive factor for severity of Escherichia coli mastitis


Table 1. Relationship between milk production loss (linear regression)/severity (logistic regression) and CL in blood and milk PMN stimulated by PMA and latex beads during experimentally induced *E. coli* mastitis. The slope and its standard error is based on the data of 15 M responders and 5 S responders cows.

<table>
<thead>
<tr>
<th>Source of PMN</th>
<th>Stimulator</th>
<th>Slope (S.E.)</th>
<th>P-value</th>
<th>Slope (S.E.)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>PMA</td>
<td>0.0044(0.0045)</td>
<td>0.34</td>
<td>0.0017(0.0008)</td>
<td>0.038</td>
</tr>
<tr>
<td>Blood</td>
<td>Latex</td>
<td>0.0041(0.0035)</td>
<td>0.26</td>
<td>0.0038(0.0025)</td>
<td>0.13</td>
</tr>
<tr>
<td>Milk</td>
<td>PMA</td>
<td>0.0185(0.0077)</td>
<td>0.029</td>
<td>0.0037(0.002)</td>
<td>0.057</td>
</tr>
<tr>
<td>Milk</td>
<td>Latex</td>
<td>0.036(0.014)</td>
<td>0.022</td>
<td>0.0062(0.0031)</td>
<td>0.043</td>
</tr>
</tbody>
</table>

Table 2. Relationship between pre-infection PMA- and –latex- stimulated blood and milk PMN CL and cfu at PIH 6 (linear regression) and during experimentally induced *E. coli* mastitis. The slope and its standard error is based on the data of 20 cows.

<table>
<thead>
<tr>
<th>Source of PMN</th>
<th>Stimulator</th>
<th>Slope (S.E.)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>PMA</td>
<td>-3.29 (1.49)</td>
<td>0.014</td>
</tr>
<tr>
<td>Blood</td>
<td>Latex</td>
<td>-3.39 (1.056)</td>
<td>0.0007</td>
</tr>
<tr>
<td>Milk</td>
<td>PMA</td>
<td>-7.91 (2.63)</td>
<td>0.0013</td>
</tr>
<tr>
<td>Milk</td>
<td>Latex</td>
<td>-16.78 (4.5)</td>
<td>0.0001</td>
</tr>
</tbody>
</table>
Table 3. Comparison of some circulating leukocytes and neutrophils as well as neutrophils, SCC and cfu in milk between moderate and severe responders to experimentally induced *E. coli* mastitis in dairy heifers. Values are means ± SEM of 15 cows (moderate) and means ± SEM of 5 cows (severe).

<table>
<thead>
<tr>
<th>PIH</th>
<th>Parameter</th>
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<th>Milk</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>M</td>
<td>S</td>
</tr>
<tr>
<td>0</td>
<td>WBC/µl</td>
<td>8209±250</td>
<td>8433±1165</td>
</tr>
<tr>
<td></td>
<td>PMN/µl</td>
<td>2412±263</td>
<td>2980±856</td>
</tr>
<tr>
<td></td>
<td>Immature N/µl</td>
<td>652±68</td>
<td>928±203</td>
</tr>
<tr>
<td>6</td>
<td>WBC/µl</td>
<td>8504±637</td>
<td>7536±2403</td>
</tr>
<tr>
<td></td>
<td>PMN/µl</td>
<td>1811±210</td>
<td>2252±722</td>
</tr>
<tr>
<td></td>
<td>Immature N/µl</td>
<td>1518±208</td>
<td>2233±410</td>
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<tr>
<td>12</td>
<td>WBC/µl</td>
<td>4271±682</td>
<td>2661±349</td>
</tr>
<tr>
<td></td>
<td>PMN/µl</td>
<td>601±141</td>
<td>273±83</td>
</tr>
<tr>
<td></td>
<td>Immature N/µl</td>
<td>1018±193</td>
<td>745±167</td>
</tr>
<tr>
<td>18-24</td>
<td>WBC/µl</td>
<td>6752±766</td>
<td>5360±578</td>
</tr>
<tr>
<td></td>
<td>PMN/µl</td>
<td>1341±231</td>
<td>776±144</td>
</tr>
<tr>
<td></td>
<td>Immature N/µl</td>
<td>2325±347</td>
<td>2263±376</td>
</tr>
<tr>
<td>&gt;48</td>
<td>WBC/µl</td>
<td>8899±324</td>
<td>11329±931</td>
</tr>
<tr>
<td></td>
<td>PMN/µl</td>
<td>2042±139</td>
<td>2825±417</td>
</tr>
<tr>
<td></td>
<td>Immature N/µl</td>
<td>1815±117</td>
<td>3205±331</td>
</tr>
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Table 4. Comparison of AUC, RLU\textsubscript{max} and T\textsubscript{max} of blood and milk PMN between moderate and severe responders during experimentally induced \textit{E. coli} mastitis. The data shown are means and standard error of 15 M responders and 5 S responders. An asterisk corresponds to a significant difference (P<0.01) between moderate (M) and severe (S) responders.

<table>
<thead>
<tr>
<th>PIH</th>
<th>Parameter</th>
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<th>PMA-stimulated PMN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Blood</td>
<td>Milk</td>
</tr>
<tr>
<td>0</td>
<td>AUC</td>
<td>6611</td>
<td>3310</td>
</tr>
<tr>
<td></td>
<td>RLU\textsubscript{max}</td>
<td>2565*</td>
<td>1097</td>
</tr>
<tr>
<td></td>
<td>T\textsubscript{max}</td>
<td>7.1</td>
<td>7</td>
</tr>
<tr>
<td>6</td>
<td>AUC</td>
<td>5739</td>
<td>3544</td>
</tr>
<tr>
<td></td>
<td>RLU\textsubscript{max}</td>
<td>2599*</td>
<td>1112</td>
</tr>
<tr>
<td></td>
<td>T\textsubscript{max}</td>
<td>7.2</td>
<td>7.2</td>
</tr>
<tr>
<td>12</td>
<td>AUC</td>
<td>4756</td>
<td>2982</td>
</tr>
<tr>
<td></td>
<td>RLU\textsubscript{max}</td>
<td>2608*</td>
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<td></td>
<td>T\textsubscript{max}</td>
<td>7.1</td>
<td>6.8</td>
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<tr>
<td>18-24</td>
<td>AUC</td>
<td>8656*</td>
<td>2370</td>
</tr>
<tr>
<td></td>
<td>RLU\textsubscript{max}</td>
<td>2315*</td>
<td>527</td>
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<td>T\textsubscript{max}</td>
<td>7.5*</td>
<td>4.8</td>
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<td>AUC</td>
<td>12294*</td>
<td>6296</td>
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<td>2947*</td>
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<td>T\textsubscript{max}</td>
<td>6</td>
<td>7.5</td>
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PMN chemiluminescence as a predictive factor for severity of Escherichia coli mastitis

Figure 1. Relationship between PMA (a, c)-and-latex (b, d) stimulated blood (a, b) and milk (c, d) PMN CL (AUC of 1000 viable PMN) prior to the inoculation of E. coli and the milk production loss at PIH 48 (n=20). Circles represent individual cows through which the regression line has been fitted. The horizontal line corresponds to the 50% milk production loss (severity threshold).
Figure 2. Comparison of pre-infection chemiluminescence (CL) kinetics of blood (a, c) and milk (b, d) stimulated with latex (a, b) and PMA (c, d) between moderate cows (n=15, dashed lines and +) and severe (n=5, solid lines and circles). The generation of CL was monitored continuously for 30 min after addition of 100 ng/ml PMA and/or 500 latex beads particles/PMN and 0.3 mM luminol to the 4.10^5 isolated cells suspension in 200µl. The curves are average of 15 M cows and 5 S cows.
Figure 3. Multiplication rate of *E. coli* (solid lines) in infected mammary glands and pattern of leukocyte influx (dashed lines) into milk in (a) severe responders (n=5) compared with (b) those in moderate responders (n=15) during experimentally induced *E. coli* mastitis.
Figure 4. Blood and milk PMN CL kinetics prior to and during experimentally induced *E. coli* mastitis in moderate (solid lines) and severe (dashed lines) responders. The number of the figures corresponds to the time post infection, with (1) for pre-infection, (2) for PIH 6, (3) for PIH 12, (4) for PIH 18, (5) for PIH 24, (6) for PIH 48 and (7) for PIH 72, whereas (a) corresponds to blood PMN with latex stimulation, (b) to milk PMN with latex stimulation, (c) to blood PMN with PMA stimulation and (d) to milk PMN with PMA stimulation. The curves are averages of 15 M and 5 S cows.
General discussion
General Discussion

Despite intense progress in veterinary medicine, dairy farm husbandry, nutrition, molecular biology and genetics, peripartum dairy cows are still susceptible -more than before- to environmental bacteria. This increased susceptibility to infectious agents has been attributed to polymorphonuclear leukocytes (PMN) function impairment (Kehrli et al., 1989; Burvenich et al., 1994; Paape et al., 1996; Shuster et al., 1996), which is maximal during early lactation (see e.g., chapter 4). Immunomodulation in high yielding dairy cows against mastitis is still far from assured. Retrospective studies together with current study reveal that bovine blood and milk PMN have the potential to produce reactive oxygen species (ROS) to eventually kill engulfed bacteria. Pathogenesis and outcome of E. coli mastitis are influenced by recruitment of PMN in milk and their survival in infection site. Many physiological, metabolic and biochemical phenomena affect on PMN function and host immunity; among them are stage-and-number of lactation and mastitis (see e.g., chapters 4 and 5). We found little information on literatures about physiological and pathological influencing factors on “milk” PMN function. This thesis was focused on the respiratory burst activity and viability of blood and milk PMN during peripartum period and mastitis. Started from bone marrow, impairment of PMN function is peculiar during periparturient period, which is multifactorial. This impairment might be cumulative when they influx to the milk. Thus, study on PMN functional changes would provide a strong cause-and-effect relationship between mastitis and periparturient period.

One of the major problems in PMN functional assay is inappropriate sampling and isolation procedure. So far, study on the quantitative role of bovine blood and milk leukocytes in healthy and mastitis cows is rare. Leukocyte differentiation in milk and blood also appears a little bit more difficult. Due to existence of variety of cells in non-mastitis milk, the cellular source of mixed cells needed to be identified. One example: the contribution of milk macrophages to PMN functional assay. This led us to conduct some fundamental assessment on standardisation of blood and milk PMN isolation, identification and quantification. To overcome any problem, in chapter 1 we particularly examined this issue and concluded that a proper PMN isolation technique simplifies PMN functional assay.

During PMN isolation of non-mastitis milk it was almost impossible to yield 100% pure PMN. It was also predictable that some of the PMN -especially milk PMN- are necrotic and functionless. So that, we further examined PMN viability assessment (chapter 2). This was done using the flow cytometric technique, applying propidium iodide exclusion method as well as microscopic observation using trypan blue exclusion technique (Colotta et al., 1992). The flow cytometric technique for PMN viability assessment was based on correct gating of PMN in the FS-SS dot plot, using PMN labeling with specific mAb for bovine PMN (Davis et al., 1987). This method was reproducible technique for rapid quantification of PMN survival in healthy and mastitis cows. PMN viability assessment would not only boost insight into the first line defense mechanism of udder, it can also facilitate further PMN functional assay e.g., PMN chemiluminescence (CL) assay.

Variety of ROS are generated during respiratory burst activity of PMN. These ROS can be quantified by the most widely used technique: CL assay (Allen et al., 1972; Weber et al., 1983; Piccinini et al., 1999; Mehrzad et al., 2000a). This sensitive CL
results from reactions of ROS with luminescence agents, which requires both an active NADPH-oxidase and an active myeloperoxidase (MPO) (Allen et al., 1972; Webb et al., 1974; Babior, 1984). Nowadays sophisticated analytical luminometers are available to study PMN CL in dairy cows. Few or little technical studies on optimised measurement conditions of bovine blood and milk PMN CL are available. For accurate PMN CL assay, CL-enhancers and activator agents and their solvents and cell aggregation preventing agents were required. It was not clear how all these factors influence the CL assay of blood and milk PMN. This was exclusively examined in chapter 3. In this chapter, a detailed description of the optimised measurement conditions of luminol-dependent CL (LDCL) technique for blood and milk PMN in high yielding dairy cows was presented. In addition, to demonstrate the contribution of blood and milk PMN to killing of invading microorganisms in the mammary gland, the relationship between PMN LDCL and intracellular killing of *S. aureus* was investigated. Both phagocytosis-induced and/or non-induced LDCL inexpensively yielded a conclusive result about PMN bactericidal efficiency. This was in agreement with Allen et al. (1972), Grebner et al. (1977) and Reeves et al. (2002), who studied on “human blood” PMN. Our study presented in chapter 3 was about milk PMN as well.

Factors that hampered the study of milk PMN were more problematic than those of blood PMN’s. For example, the variability in cellular morphology and the difficulty of cell differentiation (see chapter 1), which was necessary for CL calculation. At the start of this study, it was unclear whether macrophages isolated from bovine milk had a significant CL activity. We demonstrated that macrophages in milk produce no LDCL; so that, their interference with LDCL assay was negligible. The explanation for this lack of CL activity is that milk macrophages had no MPO activity (Chapter 4). This finding, which is in accordance with Hallén Sandgren et al. (1991), was central for the choice of the experimental set-up of milk PMN and to obtain unequivocal CL results. Indeed, the ROS from milk cells measured in LDCL assay can be solely attributed to the milk PMN, without interference of other milk cells like macrophages. This finding allowed us to quantify the respiratory burst activity of PMN obtained from non-mastitis quarters with a low SCC and variable cellular composition. Application of LDCL technique to study PMN function will surely help bovine immunologists gain more insight into first line defense mechanisms and the pathophysiology of mastitis.

One of the focuses of the thesis had been a comparative study of blood and milk PMN function and physiological influencing factors (see chapters 4 and 5). To obtain a better insight into the effects of the physiology of lactation and parity on non-specific defense mechanisms within the mammary gland, the respiratory burst activity and viability of milk and blood PMN during lactation cycle and number was investigated. To further interpretation of the PMN ROS production, it was necessary to distinguish between resting (non-stimulated) and stimulated PMN. Our studies in chapter 4 showed that CL of resting milk PMN was higher than CL of blood PMN. This might be resulted from triggering of PMN CL via ingestion of milk fat globules and casein micelles (Paape and Guidry, 1977; Dulin et al., 1988) with subsequent degranulation (Russell et al., 1976; Paape and Wergin, 1977). This pre-stimulation of milk PMN resulted in a decreased capacity to produce ROS with PMA. Together, these data indicated that milk PMN were less responsive to stimulating agents, mainly because of the pre-stimulation by milk constituents. This pre-stimulated status can sometimes
lead to a functionally exhausted PMN (Paape and Guidry, 1977; Smits et al., 2000). Diapedesis of PMN across mammary epithelium also reduces their ROS production (Smits et al., 1999). Interestingly, ROS production of resting and stimulated milk PMN was positively correlated to their counterparts in blood.

From the study in chapter 4, it was conclusive that blood PMN had higher capacity for ROS production than that of milk PMN, when stimulated. The ratio of milk to blood PMN CL was always < 1. CL of stimulated blood and milk PMN was low during early lactation. CL was much higher during mid-and late lactation. Moreover, the ratio of stimulated milk PMN to blood PMN CL was lowest immediately after calving (0.469). During mid and late lactation, however, comparable values were observed (0.782 and 0.754, respectively). The lowest responsiveness of milk PMN to PMA during early lactation could be resulted from a weaker stimulation of protein kinase C and NADPH-oxidase (Karlsson et al., 2000). This coincides with the finding that cows are more susceptible for clinical environmental mastitis (Burvenich et al., 1994; Shuster et al., 1996). This ROS production impairment of “milk” PMN indicated that some physiological factors related to the stage of lactation could be involved. We suggest that β-lactoglobulin (Mehrzad et al., 2000b), of which concentration in milk is minimal during early lactation (Caffin et al., 1984), might contribute to the “milk” PMN ROS production impairment during early lactation.

Beyond the differences in the ratio of milk to blood PMN CL, the differences were observed in the kinetics of PMN CL. LDCL kinetics were studied in blood and milk PMN. CL was measured in cows during early lactation and compared with data during mid and late lactation. PMA-stimulated blood PMN CL showed a biphasic course during mid and late lactation (immediate peak at 3 min and delayed peak at 12 min). During early lactation only an immediate peak was observed. CL in milk PMN was monophasic as well as smaller than in blood. The shape of the kinetics events during PMA-induced LDCL reveals some details on the location of the ROS that are produced intra-and-extracellularly (Rosen and Klebanoff, 1976; DeChatelet et al., 1982; Briheim et al., 1984). The LDCL kinetics after 3-4 minutes is considered to be the result of intracellular events (DeChatelet et al., 1982; Edwards et al., 1986; Faulkner et al., 1993). It was conclusive that the intracellular H$_2$O$_2$ production is higher in blood than in milk PMN. According to the studies in chapter 4, it can be concluded that myeloperoxidase- H$_2$O$_2$-halide system, a critical part of the physiological mechanism for bactericidal activity, is impaired during early lactation. The impaired PMN bactericidal activity during early lactation (chapter 3) was also reported elsewhere (Dosogne et al., 2001). The absence of a second peak in milk PMN throughout lactation cycle and in blood PMN immediately after calving is warranted to evaluate the role of PMN in in vivo bactericidal capacity of mammary gland. Figure 1 shows an overview of blood and milk PMN CL kinetics.
Figure 1. A comparison between blood and milk PMN CL kinetics during early lactation (dashed lines) and mid-and-late lactation (solid lines) studied in the thesis. The intra-and-extra-cellular ROS production is overviewed. Throughout lactation cycle, the kinetics of milk PMN CL never exhibited double phase patterns. Blood PMN CL kinetics immediately after calving gave neither double phase nor high intensity as in mid-and late lactation.

In chapter 5, we demonstrated that the relative magnitude of blood and milk PMN CL impairment differed from different number of lactation in dairy cows. We examined blood and milk PMN LDCL in primiparous (first calving) and pluriparous (fourth to fifth calving) during late pregnancy, around parturition and early lactation. Blood PMN CL in pluriparous cows showed a sharper decrease. Our results also indicated that the impairment of blood PMN CL in primiparous and pluriparous dairy cows during peripartum period was confined to the period between periparturient week (ppw) -1 and 2. This is in accordance with chapter 4 and (Moreira da Silva et al., 1998; Kimura et al., 1999; Hoeben et al., 2000a), indicating that the transitional period from parturition to lactation is immunosuppressive. This can lead to an overall impaired bactericidal capacity of blood PMN during early lactation. After periparturient day (ppd) 7, blood PMN CL recovery rate was faster in primiparous cows. At ppw 1, milk PMN CL was also minimal both in primiparous and pluriparous cows. Since lower PMN ROS production increases the probability of developing clinical mastitis (Zecconi et al., 1994; Shuster et al., 1996), the lower “milk” PMN CL in pluriparous dairy cows during early lactation would result, in part, in a higher probability of developing clinical mastitis in pluriparous cows. Further epidemiological research is needed, however, to confirm the link between age and severity of mastitis.

The kinetics of cellular CL of blood and milk PMN showed further “novel” information about differences between the primiparous and pluriparous cows. The CL kinetics of blood PMN at ppd -2 and 2 and of milk PMN at ppd 2 exhibited different responses to PMA, with higher intensity and durability, peaking and subsiding more slowly in primiparous dairy cows. In addition to a longer onset time for primiparous blood PMN CL at ppd -2 and 2, the PMA-stimulated CL at ppd -2 was cumulatively remained for at least 13 min in primiparous cows, whereas it was only maintained for
less than 5 min in pluriparous cows. Furthermore, the PMA-stimulated CL at ppd 2 was remained increasing for at least 11 min in primiparous cows, while in pluriparous it was only peaked around 4 minutes and quickly subsided. This lower CL plateau could lead to an inefficient “intracellular” bactericidal activity of blood PMN around parturition in pluriparous dairy cows. The slightly higher intensity and peak in primiparous milk PMN CL suggests that the diminished oxygen-dependent intracellular killing of milk PMN against pathogens was more pronounced in pluriparous cows.

Flow cytometric studies revealed little effect of lactation cycle and number on viability of blood PMN (chapters 4 and 5). In contrast, the viability of milk PMN was substantially influenced by stage of lactation and parity. This viability was far lower during early lactation than during mid- and late lactation. This was the first report ever and was later confirmed by Van Oostveldt et al. (2001). The mechanism for decreased survival of milk PMN during early lactation remains unknown. Based on studies with human and bovine PMN (Mayer et al., 1989; Jankowski et al., 2002), the contribution of PMN NADPH-oxidase activity to viability appears critical. In addition, there was a strong positive correlation between PMA-induced milk PMN CL and their viability (chapter 3). PMA is a potent NADPH-oxidase and protein kinase-C agonist (Karlsson et al., 2000). As the NADPH-oxidase is a trigger of PMN respiratory burst and proton transportation into the PMN phagosomal and cytosolic space, the enzyme’s activity is regulated by MPO as well (Edwards et al., 1986). The lower resting and stimulated milk PMN CL during early lactation (chapter 4) indicated NADPH-oxidase and MPO activity impairment. NADPH-oxidase activity contributes to phagosomal and cytosolic pH homeostasis (Reeves et al., 2002), maintaining PMN stoichiometry and electroneutrality (Takanaka et al., 1988). This evidence supports the conception of rapid and profound cytosolic acidification, which could result in a faster PMN necrosis and thus might have had an effect on PMN survival in our study.

Milk PMN viability impairment was more pronounced in pluriparous dairy cows during early lactation. This observation contrasts with the viability of blood PMN, which was consistently ~100 %. The minimal milk PMN viability was also most pronounced within the first week postpartum. This is in accordance with (chapter 4). The exact mechanism for higher survival of milk PMN in primiparous dairy cows and/or their more pronounced impairment in pluriparous cows remains unclear. Apart from above explanations, there could also be other physiological factors, such as recruitment of younger neutrophils in the milk compartment of primiparous cows, which might involve the delay of apoptosis and the increase of viability. The consistently higher milk PMN viability in primiparous cows suggests that primiparous cows might be a better source of milk PMN for neonates and in vitro experiments.

The lactation-and-parity-related “milk” PMN viability was interesting issue. Higher milk PMN viability could contribute to a better protection of mammary gland against invading pathogens during mid-and late lactation. Moreover, higher milk PMN viability in primiparous dairy cows could provide a strong protection for mammary gland against bacterial pathogens. The latest research in human (Vissers et al., 2001) uncovers the ability of intracellular vitamin C to protect PMN from apoptosis and necrosis. Also, GHS’s ability to act as a two-electron reductant for a wide variety of ROS has led a great deal of scientists to investigate its role as PMN protection from necrosis. This protection is resulted from altering caspase activity (Fadeel et al., 1998;
Akgul et al., 2001), as caspase activation is a known apoptosis-processing singnaling pathway. Current knowledge about milk PMN viability reveals no such examinations have been conducted in bovine. For example, a comparative study of vitamin C in milk and blood PMN in high yielding dairy cow may provide further evidence about lower viability of milk PMN. The question of whether vitamin C concentration and GHS loss in milk PMN compare to the blood’s, particularly in early lactation (primi- and-pluriparous) cows, is valuable to be answered. In healthy cow the most interactive approaches of PMN viability would be interaction between ROS generation and caspase activity of milk PMN. This is warranted to further research.

To further evaluate the blood and milk PMN functional changes, experimentally induced mastitis conducted in chapters 6, 7 and 8. The endotoxin-induced mastitis (chapter 6) and E. coli mastitis (chapter 7) showed that the viability of PMN isolated from milk of both endotoxin-and E. coli injected quarters increased substantially. The viability of non-injected quarters also increased significantly at post-infection hours (PIH) 6 and 12. Viability was higher in PMN from inflamed quarters than from non-inflamed quarters. Pre-challenge values were obtained at PIH >72. The viability of PMN isolated from blood did not change significantly during both endotoxin and E. coli mastitis. The modulation of bovine PMN survival in milk by E. coli and/or endotoxin could be linked to the physiopathology of E. coli-induced mastitis, especially the kinetics of PMN diapedesis through the blood/milk barrier. The precise mechanism of increased milk PMN viability during mastitis is still debatable. One concern can be that the anti-apoptotic effect of cytokines e.g., TNF-α, IL-1 and IL-6 or bacterial endotoxins (Colotta et al., 1992; Lee et al., 1993; Sweeney et al., 1998). Milk concentrations of all of these host-and pathogen derived cytokines are extremely high throughout day 1 of E. coli and endotoxin mastitis (Shuster et al., 1993; Blum et al., 2000; Hoeben et al., 2000b; Hagiwara et al., 2001). As CL reveals, contribution of NADP-oxidase to cytosolic pH homeostasis can be an attributable factor to milk PMN viability modulation; this was explained earlier. There could also be other systemic and local factors, such as recruitment of younger PMN (see chapters 6 and 7) in the endotoxin and/or E. coli inflamed quarters, involved in increasing viability. The fast diapedesis also explains the delayed apoptosis and increased viability of PMN at the site of infection (Ferrante, 1992; Lee et al., 1993; Watson et al., 1997). The contribution of blood-milk barrier (Van Oostveldt et al., 2002) and mammary gland injury (Sladek et al., 2001) in the modulation of PMN apoptosis can also be the attributable factors. In non-infected quarters, the increased milk PMN viability emphasizes the systemic effect of endotoxin and/or E. coli mastitis on milk PMN survival. E. coli and/or endotoxin-challenged quarters’ PMN viability and their CL were interrelated, potentially offering more effective first line defense for mammary gland during infection. Throughout endotoxin and/or E. coli mastitis, increased viability of inflamed quarters’ PMN boosted mammary gland immunity against pathogens, terminating inflammation in vivo. The mastitis-related milk PMN viability modulation, which can prevent inappropriate or prolonged inflammation, warrants further investigation. Tables 1 and 2 give a general overview about blood and milk PMN functional changes during physiological and mastitis conditions studied in this thesis.
General discussion

Table 1. An overview of blood PMN function changes during physiological and mastitis conditions studied in the thesis; ppw = periparturient week, M = moderate, S = severe, - = normal or unchanged, ↑ = increased, ↑↑ = strongly increased, ↓ = decreased, ↓↓ = strongly decreased. Primiparous compared to pluriparous cows; the data from mastitis is 6-12 h after mastitis induction.

<table>
<thead>
<tr>
<th>Cow conditions</th>
<th>CL</th>
<th>Viability</th>
<th>Diapedesis</th>
<th>Immaturity</th>
</tr>
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<tbody>
<tr>
<td><strong>Physiological</strong></td>
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<tr>
<td>Early lactation</td>
<td>↓</td>
<td>-</td>
<td>↓</td>
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<td>Mid lactation</td>
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<tr>
<td>Late lactation</td>
<td>-</td>
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<tr>
<td>Primiparous (ppw –1 to 1)</td>
<td>↑</td>
<td>-</td>
<td>↑</td>
<td>↑</td>
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<tr>
<td>Pluriparous (ppw –1 to 1)</td>
<td>↓</td>
<td>-</td>
<td>↓</td>
<td>-</td>
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<tr>
<td><strong>Mastitis</strong></td>
<td></td>
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<tr>
<td>Endotoxin</td>
<td>↑↑</td>
<td>-</td>
<td>↑↑</td>
<td>↑</td>
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<tr>
<td>E. coli (M responders)</td>
<td>↑↑</td>
<td>-</td>
<td>↑↑</td>
<td>↑</td>
</tr>
<tr>
<td>E. coli (S responders)</td>
<td>↑</td>
<td>-</td>
<td>↑</td>
<td>↑↑</td>
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Table 2. When PMN recruit into mammary gland, their functions change; these changes differed during physiological and mastitis conditions of cows. Here some changes found in our study are summarised. ppw = periparturient week, M = moderate, S = severe, - = normal or unchanged, + = changed, +/- = but are to be studied, ↑ = increased, ↑↑ = strongly increased, ↓ = decreased, ↓↓ = strongly decreased. Primiparous compared to pluriparous cows; the data from mastitis is 6-12 h after mastitis induction.

<table>
<thead>
<tr>
<th>Cow conditions</th>
<th>CL</th>
<th>Viability</th>
<th>Structure</th>
</tr>
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<tbody>
<tr>
<td><strong>Physiological</strong></td>
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<tr>
<td>Early lactation</td>
<td>↓↓</td>
<td>↓↓</td>
<td>+</td>
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<tr>
<td>Mid lactation</td>
<td>↓</td>
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<tr>
<td>Late lactation</td>
<td>↓</td>
<td>↓</td>
<td>+</td>
</tr>
<tr>
<td>Primiparous (ppw –1 to 1)</td>
<td>↓</td>
<td>↓</td>
<td>+/-</td>
</tr>
<tr>
<td>Pluriparous (ppw –1 to 1)</td>
<td>↓↓</td>
<td>↓↓</td>
<td>+/-</td>
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<tr>
<td><strong>Mastitis</strong></td>
<td></td>
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</tr>
<tr>
<td>Endotoxin</td>
<td>↑↑</td>
<td>↑↑</td>
<td>+/-</td>
</tr>
<tr>
<td>E. coli (M responders)</td>
<td>↑↑</td>
<td>↑↑</td>
<td>+/-</td>
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<td>E. coli (S responders)</td>
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Analogous with viability, the stimulated milk PMN CL of endotoxin and/or E. coli-injected quarters increased substantially at PIH 6 to 24 and slightly in non-inflamed quarters (see chapters 6 and 7). There were also some changes on blood PMN CL, which is consistent with previous studies (Heyneman et al., 1990; Vandeputte-Van Messom et al., 1993). Paralleled to stimulated PMN CL, non-stimulated milk PMN of inflamed quarters also showed substantially higher ROS production than those of blood and non-inflamed quarters. This can be due to increased production of host and/or pathogen-derived cytokines in infected quarters (Hoeben et al., 2000b; Blum et al., 2000; Hagiwara et al., 2001). The detailed information on non-stimulated and
PMA and/or latex-induced blood and milk PMN CL “kinetics” would be more interesting. This again uncovered intra-and-extracellular ROS generation during course of endotoxin or *E. coli* mastitis. The slightly increase of CL intensity and durability in PMN of non-infected quarters showed a systemic effect of endotoxin and/or *E. coli* mastitis in dairy cows. The kinetics of PMA-stimulated blood and milk PMN CL revealed that the intensity and duration of milk PMN CL from endotoxin and/or *E. coli*-injected quarters increased markedly at PIH 6 to 12. Contrary to the pre-challenge values, the intensity and the duration of ROS production of milk PMN from non-injected quarters was also increased. The CL intensity of milk PMN from inflamed quarters was exceedingly higher than of blood PMN and of milk PMN from non-inflected quarters at PIH 6 to 12. Indisputably, intracellular H$_2$O$_2$ production, which is precursor of variety of subsequent powerful ROS (Chapman et al., 2002), is maximal in PMN from endotoxin and/or *E. coli*-infected quarters at PIH 6, 12 and 18 (chapters 6 and 7). The shape of latex stimulated CL of PMN of *E. coli* infected quarters emphasized increased phagocytosis activity. The higher phagocytosis activity boosted exposure of *E. coli* to intracellular ROS during early phase of infection. As a result, the phagolysosome of PMN uses MPO-H$_2$O$_2$-Cl system to chlorinate bacterial proteins (Rosen et al., 2002), thereby eliminating phagocytosed *E. coli*. Certainly, the exclusive milk PMN CL shape during early phase of mastitis revealed the quality of milk PMN, because the milk PMN had maximal viability as well. Therefore, timely PMN ROS production enabled PMN to efficiently kill and detoxify *E. coli* and endotoxin. Figure 2 shows an overview of our findings about changes in PMN CL, viability and dynamic of PMN structure and maturity in healthy and mastitis dairy cows.

![Figure 2](image_url)

**Figure 2.** An schematic overview on our findings (together with literatures) about changes in PMN MPO-H$_2$O$_2$-Halide system (Solid lines), viability (dashed lines) and dynamic of PMN structure and maturity in healthy and mastitis (6-12 hours after endotoxin and/or *E. coli* induction) dairy cows. Formed in bone marrow, blood PMN function and structure changed after normal diapedesis in milk. These changes differed during mastitis. The most probable reason for these disparities would be the “rate of diapedesis”, which is faster during mastitis.
Conversely, PMN ROS is a “double-aged sward”. For example, throughout endotoxin or *E. coli* mastitis, maximal milk PMN CL at PIH 48 or 72 would be counterproductive. So, higher milk PMN CL at PIH 6 would be far more important than at PIH e.g. 72. This alleviates the severity of inflammation. Undoubtedly, when bacterial invasion occurs, PMN ROS production will facilitate pathogen elimination. When ROS is produced adequately, cell and tissue damage will be far less than when it is inadequate. Uncontrolled generation of ROS is harmful for many cell systems. For example, T cell hyporesponsiveness and lymphocyte proliferation inhibition caused by ROS (Nonnecke and Harp, 1988; Cemerski et al., 2002). On the other hand, blood and milk PMN by phagocytosis of e.g. *S. aureus* inhibits lymphocyte cytotoxicity and enhances mononuclear cell viability (Nonnecke and Harp, 1988). ROS also differently enhances natural killer cell and T cell activity (Suthanthiran et al., 1984; Cemerski et al., 2002). Overall, PMN ROS may not only damage to the cells and tissues but can also accelerate recovery of mastitis. This can be pinpointed by examining the kinetics of PMN ROS production.

The contribution of residual milk PMN on the severity of infection was examined in chapter 8. In this chapter, we investigated the impact of pre-and early phase of infection blood and milk PMN CL on the mastitis severity risk. Concurrently, the shape of SCC (as an index for PMN diapedesis) and cfu fluctuations during course of *E. coli* mastitis was examined. Cows were identified in moderate (M) and severe (S) responders accordingly. In M responders, the pre-and early phase of infection blood and milk PMN CL was significantly higher than those of S responders. Both in blood and milk, by increasing CL the severity risk decreased. Figure 3 shows overall disparities of milk PMN CL, PMN diapedesis rate and cfu dynamics in *E. coli* infected quarters between M and S responders. The shape of milk PMN CL, SCC and cfu fluctuations indicated that the fast increased milk PMN CL and SCC during infection lags exponential growth of *E. coli*, facilitating recovery of coliform mastitis in M responders. In contrast, neither CL nor SCC seemed capable of timely declining cfu in S responders. This result is consistent with the hypothesis that the ability to recruit PMN to mammary gland during early hours of mastitis is important in limiting the severity of the gland injury. Blood PMN response to PMA and/or latex beads didn’t decline substantially during first day of infection in M cows, whereas in S group this decline was substantial. Indeed, in S group rebound effect was observed, both for CL and hematopoeigenesis (see chapter 8). This was in accordance with (Heyneman et al., 1990; Vandeputte-Van Messom et al., 1993).
We also focused on PMN CL kinetics and severity of mastitis. What made the M responders much stronger against *E. coli* mastitis was not merely the result of absolute CL values, but also of PMN CL kinetics before and during *E. coli* infection. To our knowledge, little is known about the links of milk or blood PMN CL kinetics to the severity of *E. coli* mastitis. Application of uncoated latex beads in our study was implying to non-specific phagocytosis. From our study in chapter 8 some important conclusions can be made: First, blood PMN and the newly migrated PMN in milk are consistently more efficient in M responders. Indeed, from PIH -24 to 24 the RLU_{max} in blood and milk PMN was always higher in M cows than in S ones. Similarly, the plateau and T_{max} of both CL curves were higher in M responders. This results from higher overall oxidation-reduction reactions in blood and milk PMN of M cows. Secondly, T_{max} of latex stimulated blood PMN CL kinetics at PIH 18-24 demonstrated that latex beads are phagocytosed incompletely by PMN of S cows. So, T_{max} of particle stimulated CL kinetics gave further explanation about functional status of PMN in M and S responders. The highest affinity of PMN to uptake latex beads in M responders could partly give an idea about activity of actin filaments and myosin, which are crucial during phagocytosis (Cox et al., 2002). For example, microtubules in PMN play central role in membrane invaginations, phagosome formation and internalization of phagosome during phagocytosis (Hoffstein et al., 1977). For the principal mechanism of engulfing *E. coli* by post-diaptetic PMN in milk, rearrangement of cytoskeleton and pseudopod extension would be pivotal. Thirdly, the impairment of intracellular reactions of MPO–H₂O₂ halide system is more pronounced in S cows. The decreased intracellular events of ROS production could be the main cause of lower milk PMN CL and intracellular bactericidal efficiency in S responders at PIH 6, 12, 18 and 24. This resulted in extremely higher cfu in milk of S responders after *E. coli* infection. Throughout *E. coli* mastitis, the appearance of “fast-strong local” response in M cows and of “delayed-weak local” response in S cows was responsible for most of the disparities between M and S cows.
Conclusions

To evaluate the importance of PMN function for mammary gland defense against pathogens, cow factors that could influence on first line defense of the gland were studied. Impact of stress from parturition, lactation and mastitis on PMN functions (CL, viability -only milk- and diapedesis) and consequently on severity of coliform mastitis was critical. Blood PMN CL impairment coincided with milk PMN CL impairment; this was maximal immediately after calving, partly revealing reason of higher mastitis incidence during early lactation. The parturition-related blood and milk PMN CL impairment was more pronounced in older dairy cows. PMN ineffectiveness against invading pathogens was not merely resulted from the quantity of PMN, but, more importantly, from the quality of PMN, which was identified via PMN CL kinetics and PMN viability. In healthy cows the lowest milk PMN quality was found during early lactation, which was more pronounced in older cows, proving one more reason for high susceptibility of dairy cows to mastitis during early lactation.

To demonstrate the crucial role of blood and milk PMN on cow- E. coli interactions, the pre-infection blood and milk PMN CL was pivotal for cow’s response against E. coli invasion in the mammary gland. This, together with maximal milk PMN CL and viability and fast diapedesis during early phase of infection accelerated bacterial removal and recovery of E. coli mastitis. These three phenomena (CL, viability and diapedesis) revealed that in M cows the “fast-local responses” were strongly efficient. This high efficiency prevented inappropriate and prolonged inflammation and halted further harmful systemic responses.

Our findings aimed to increase the insight into the first line defense mechanism of mammary gland, could further deepen our understanding at the complex physiopathology of mastitis and other early lactation-related infections. It is conceivable, however, more novel findings on these topics remain for future research.

Future prospectives

The issues on peripartum dairy cows are many, among them is PMN function that has been the researchers’ past, current and future concern. With the knowledge obtained in this thesis, it is clear that peripartum high yielding dairy cows are relatively immunosuppressed. This could boost cow’s susceptibility to environmental bacterial infections. Nowadays, peripartum dairy cows are more susceptible to environmental pathogens than before. The main concern now is how to control and enhance these non-specific aspects of immune system. Clearly, the most appropriate treatment for E. coli mastitis is preventive treatment. Our study showed the severity of mastitis is highly related to pre-infection PMN CL, quick recruitment of PMN in milk and their viability. All of these PMN functions impaired from ppw -2 to 2. Therefore, preventive measures on dairy herd around parturition should be thoroughly performed. The preventive measures should be aimed at lowering stressful conditions and ensuring a high standard of hygiene at ppd -2 to 2. As “high yielding” is highly involved in PMN impairment, genetic measures aim for optimal -not maximal; for slightly low yield- production must be implemented for the future.
Immunomodulation against mastitis is still far from guarantee. The long-term, environmentally friendly ways and fundamental solution for early lactation-related infectious diseases is “to strengthen their first line defense” by means of attainable physio-immunological approaches. This requires more insight into the first line defense mechanism, which is absolutely crucial. Perhaps one exciting and environmentally acceptable approach for mastitis control would be application of “probionts”. Protection of mammary gland from pathogens with less/non-pathogen bacteria, then further research on host-bacteria interactions would be promising.

To uncover further evidence on PMN impairment around parturition the shape of blood and milk PMN CL proven one more reason for high susceptibility of dairy cows to mastitis. On this topic, many more questions remain open for future mastitis research. Future research is also necessary to pinpoint the physiopathological influencing factors on milk PMN viability. The hypotheses of contribution of vitamin C, GHS and dynamic of phagosomal pH on PMN viability and first line defense around parturition could be tested in the future research.

As our study also revealed, the protruding psuedopodes in blood differs from those of milk; it would be worth studying the impact of lactation cycle, parity and mastitis on surface morphology of blood and milk PMN. To further mimic the first line defense mechanism similar study should be conducted on “bone marrow-blood-barrier”. Positive role of transient PMN impairments during peripartum period should not be ignored, because this might be responsible for less tissue damage during periparturient period.

Because younger dairy cows had stronger first line defense mechanism, the possibility of fast-self recovery from environmental pathogens would be far higher than old dairy cows. Thus, younger cows would require less prophylactic and therapeutic antibiotics for mastitis prevention. This suggests encouraging farmer cull dairy cows older than 4 years from the herd; though expensive, the social concerns of “potential food scares” would be alleviated. “Ruminant” mammary gland is also responsible for providing immunity to neonates from invading pathogens. The higher quality of milk PMN in younger cows might offer more efficient maternal immunity to “neonates”. This also opens new hopes for future research in ruminant immunology. Study should also be focused on molecular mechanisms of higher blood and milk PMN functions in heifers.
References


General discussion


Summary
Summary
Summary

This thesis focuses on the respiratory burst activity and viability of blood and milk polymorphonuclear leukocytes (PMN) during the periparturient period and mastitis. The major general problems encountered in the PMN functional assay are inappropriate sampling, artifacts caused by isolation and the complexity of differentiation (especially in milk). In part 1 of the thesis we dealt with these analytical/methodological issues and concluded that appropriate PMN isolation technique followed by a flow cytometric PMN viability assay, was rapid and inexpensive and yielded correct and reproducible results (chapter 1 and 2). In addition, the factors that hampered PMN chemiluminescence (CL) assay were also more problematic for milk than blood (a detailed description of influencing factors on blood and “milk” PMN CL assay was reported in chapter 3). Finally, the method for blood and “milk” PMN CL assay was optimized and simplified. In brief, both the phagocytosis-induced and the non-induced luminol-dependent CL data yielded conclusive results about the PMN bactericidal efficiency in blood and milk.

In chapters 4 and 5 of this thesis our aim was to carry out a comparative study of blood and milk PMN CL and viability under physiological influencing factors. To the best of our knowledge, we were the first to demonstrate the influence of lactation cycle and parity on blood and milk PMN CL and viability. The CL of “resting” milk PMN was systematically higher than that of blood PMN. However, when stimulated, blood PMN had a higher reactive oxygen species (ROS) production capacity than milk PMN, indicating that milk PMN are less responsive to stimulation. The ratio of stimulated milk to blood PMN CL was always < 1, being minimal during early lactation. CL of stimulated blood and milk PMN was also lowest during early lactation compared to that in mid-and late lactation. PMN CL differences were also observed in their kinetics. Blood PMN CL showed a biphasic course during mid-and late lactation (immediate peak at ~4 minutes and delayed peak at ~12 minutes following PMA stimulation). Remarkably, during early lactation only an immediate peak was observed. The CL of milk PMN was always monophasic. These CL kinetics changes revealed an impaired myeloperoxidase-H2O2-halide system during early lactation, resulting in PMN bactericidal inefficiency. During the periparturient period, the magnitude of blood and milk PMN CL impairment was more pronounced in pluriparous dairy cows than in primiparous cows. This suggests that during early lactation pluriparous dairy cows would develop more severe clinical mastitis. Flow cytometric studies revealed no effect of lactation cycle and number on the viability of blood PMN. In contrast, the viability of milk PMN was influenced by stage of lactation and parity, again being lower during early lactation than during mid-and late lactation. The higher milk PMN viability potentially contributes to a better protection of the mammary gland against invading pathogens during mid-and late lactation. Milk PMN viability impairment was less pronounced in primiparous dairy cows, suggesting a stronger protection of the mammary gland against invading pathogens.

In the last part of the thesis the role of PMN function on the pathogenesis of endotoxin mastitis (chapter 6) and *E. coli* mastitis (chapter 7) was studied. The stimulated milk PMN CL of endotoxin and/or *E. coli*-injected quarters increased substantially at post-infection hours (PIH) 6 to 24 and slightly in non-inflamed quarters. Less extensive changes were observed on blood PMN CL. Non-stimulated milk PMN of inflamed quarters also showed a substantially higher ROS production.
than those of blood and non-inflamed quarters. The detailed CL changes were confirmed in non-stimulated and PMA and/or latex-stimulated blood and milk PMN. CL kinetics showed that the intensity and duration of milk PMN CL from endotoxin and/or E. coli-injected quarters increased markedly at PIH 6 to 12. Moreover, the CL intensity and duration of milk PMN from inflamed quarters exceeded that of blood and of milk PMN from non-inflamed quarters. These findings indicate that the intracellular H$_2$O$_2$ production and phagocytosis activity is maximal in PMN from endotoxin and or E. coli-infected quarters. Concomitantly, the viability of PMN from endotoxin- and E. coli injected quarters also showed a marked increase. Though less extensive, the PMN viability in non-infected quarters also increased significantly at PIH 6 and 12. In contrast, the viability of blood PMN did not change significantly during endotoxin and E. coli mastitis. Throughout endotoxin and/or E. coli mastitis, the increased PMN CL and viability in inflamed quarters is probably counteracting the udder infection in vivo.

Finally, in chapter 8 we investigated the impact of pre-and early phase infection blood and milk PMN CL on mastitis severity. The course of somatic cell count (SCC) and colony forming unit (cfu) fluctuations during E. coli mastitis was examined. Cows were accordingly classified in moderate (M) and severe (S) responders. In M responders, the blood and milk PMN CL was significantly higher than in S responders during the pre- and early phases of infection. A negative correlation was found between blood and milk PMN CL and mastitis severity. The fast increase in milk PMN CL and SCC during infection resulted in a diminished cfu and an accelerated recovery of coliform mastitis in the M responders. In contrast, the increased PMN CL and SCC in infected quarters were inadequate to control the cfu in S responders. What made the M responders’ defense stronger against E. coli mastitis was not merely the higher PMN CL but also the different PMN CL kinetics before and during E. coli infection. To the best of our knowledge, we were the first to report the link of milk or blood PMN CL kinetics to the severity of E. coli mastitis. We hypothesize that a suppressed intracellular ROS production could be the main cause of the impaired milk PMN bactericidal efficiency in S responders.

Investigation of the underlying biochemical mechanisms explaining our innovative findings could, therefore, further deepen our understanding of the complex physiopathology of mastitis and other early lactation-related infectious diseases in the high-yielding dairy cow.
Samenvatting
(Dutch summary)
Samenvatting

In dit proefschrift werden de ‘oxidatieve burst’ en de viabiliteit van polymorfonucleaire leukocyten (PMN), geïsoleerd uit melk en bloed van koeien rond de partus en tijdens mastitis, bestudeerd.

Uit de literatuur blijkt dat de belangrijkste problemen bij het bestuderen van PMN-functies zich vooral situeren op het niveau van een adequate bemonstering en artefacten ten gevolge van de isolatie. Ook de complexiteit van de differentiatie van cellen blijkt afhankelijk van het gebruikte medium, een probleem te zijn.

In het eerste deel van dit proefschrift onderzochten we bovenstaande analytische en methodologische problemen. Het onderzoek wees uit dat een aangepaste isolatietechniek, gevolgd door een flowcytometrische viabiliteitsmeting, niet alleen tot een goede en reproduceerbare isolatie leidt maar tevens relatief snel kan uitgevoerd worden (hoofdstuk 1 en 2). Daarnaast is ook gebleken dat de bepaling van de chemiluminescentie sterk wordt beïnvloed door tal van factoren. Dit is vooral het geval voor PMN afkomstig van melk. Deze factoren werden gedetailleerd beschreven in hoofdstuk 3. De methode voor de meting van de chemiluminescentie van bloed en melk PMN werd geoptimaliseerd en vereenvoudigd. Op die wijze leverden zowel de fagocytose gemedieerde als de niet-fagocytose gemedieerde luminol afhankelijke chemiluminescentie, betrouwbare resultaten op.

In hoofdstuk 4 en 5 lag het in onze bedoeling de invloed van verschillende fysiologische parameters en situaties op de chemiluminescentie en de viabiliteit van melk PMN te vergelijken met hun invloed op bloed PMN. De invloed van het lactatiestadium en de pariteit op chemiluminescentie en viabiliteit werd nooit eerder bij koeien bestudeerd. De chemiluminescentie van niet gestimuleerde melk PMN was steeds hoger dan de chemiluminescentie van PMN afkomstig van bloed. Na stimulatie echter, werd voor bloed PMN een grotere capaciteit om reactieve zuurstofradicalen (‘reactive oxygen species’, ROS) te vormen, gevonden. Dit wijst erop dat melk PMN minder gevoelig zijn voor stimulerende agentia. De verhouding van de chemiluminescentie van melk PMN ten opzichte van deze van bloed was steeds kleiner dan 1 en was bovendien minimaal tijdens de vroege lactatie. De chemiluminescentie was veel hoger tijdens gevorderde en late lactatie.

De verschillen in chemiluminescentie van PMN afkomstig van bloed en melk uitten zich ook in hun kinetiek. De chemiluminescentie van bloed PMN vertoonde een bifasisch verloop gedurende gevorderde en late lactatie (een onmiddellijke piek op ~ 4 minuten en een uitgestelde piek op ~ 12 minuten na ‘phorbol-12-myristate-13-acetate, PMA’-stimulatie). Gedurende vroege lactatie werd evenwel enkel een ogenblikkelijke piek vastgesteld. Voor wat de melk PMN betreft, werd voor alle lactatiestadia een monofasisch verloop vastgesteld. Deze chemiluminescentie kinetiek onthult een verstoord myeloperoxidase-H2O2-halide systeem tijdens vroege lactatie dat verantwoordelijk kan zijn voor een inefficiëntie in de bacteriedodende capaciteit van PMN.

De verstoring in chemiluminescentie van bloed en melk PMN tijdens vroege lactatie was meer uitgesproken in pluripare koeien dan in primipare koeien. Dit doet veronderstellen dat pluripare koeien meer kans hebben een ernstige vorm van
Samenvatting

Klinische mastitis te ontwikkelen tijdens vroege lactatie hetgeen bevestigd wordt in de praktijk.

Aan de hand van flowcytometrische technieken hebben geen invloed van het lactatiestadium of het aantal kalvingen op de viabiliteit van bloed PMN aan het licht kunnen brengen. De viabiliteit van melk PMN daarentegen werd wel beïnvloed door het lactatiestadium en pariteit. Ook hier was de viabiliteit lager in vroege lactatie dan in gevorderde of late lactatie. De hogere viabiliteit van melk PMN in gevorderde en late lactatie kan een indicatie zijn voor een betere bescherming van de melkklier tegen pathogenen tijdens deze lactatiestadia. De verstoring van de viabiliteit van melk PMN in vroege lactatie was minder uitgesproken bij primipare koeien wat doet veronderstellen dat deze koeien een betere bescherming tegen pathogenen genieten.

In het volgende deel van dit proefschrift werd de rol van PMN in de pathogenese van endotoxine mastitis (hoofdstuk 6) en *E. coli* mastitis (hoofdstuk 7) bestudeerd.

De chemiluminescentie van met PMA gestimuleerde PMN, afkomstig van melk van met endotoxine en/of *E. coli* geïnjecteerde kwartieren, steg aanzienlijk 6 tot 24 uur na infectie. De chemiluminescentie van melk PMN afkomstig van de niet geïnfecteerde kwartieren was licht verhoogd. De veranderingen in de chemiluminescentie van bloed PMN waren minder uitgesproken. Melk PMN van geïnfecteerde kwartieren vertoonden zonder PMA-stimulatie ook een aanzienlijk hogere ROS-productie dan bloed PMN en PMN afkomstig van niet geïnfecteerde kwartieren. De kinetiek van de chemiluminescentie toonde aan dat de intensiteit en de piek van de chemiluminescentie van bloed PMN afkomstig van melk PMN overschreden bovendien de waarden van bloed en melk PMN van niet ontstoken kwartieren. Deze bevindingen wijzen erop dat de intracellulaire H₂O₂-productie en fagocytose maximaal is in PMN afkomstig van melk van endotoxine en/of *E. coli* geïnjecteerde kwartieren. De viabiliteit van melk PMN afkomstig van endotoxine en *E. coli* geïnjecteerde kwartieren vertoonden eveneens een duidelijke stijging. De viabiliteit van PMN afkomstig van de niet geïnfecteerde kwartieren was hoger van 6 tot 12 uur na infectie. De intensiteit en de duur van deze piek in de chemiluminescentie van melk PMN overschreden bovendien de waarden van bloed en melk PMN van niet ontstoken kwartieren. Deze bevindingen wijzen erop dat de intracellulaire H₂O₂-productie en fagocytose maximaal is in PMN afkomstig van melk van endotoxine en/of *E. coli* geïnjecteerde kwartieren. De viabiliteit van melk PMN afkomstig van endotoxine en *E. coli* geïnjecteerde kwartieren vertoonden eveneens een duidelijke stijging. De viabiliteit van PMN afkomstig van de niet geïnfecteerde kwartieren was hoger van 6 tot 12 uur na infectie alhoewel dit minder uitgesproken was. De viabiliteit van de bloed PMN daarentegen wijzigde niet significant tijdens endotoxine en *E. coli* mastitis. De chemiluminescentie en viabiliteit van PMN afkomstig van geïnfecteerde kwartieren limiteert wellicht de infectie en bijgevolg ook de weefselbeschadiging.

In het laatste hoofdstuk (hoofdstuk 8) tenslotte, bestudeerden we de relatie tussen de chemiluminescentie van bloed en melk PMN en de ernst van mastitis in de periode voor infectie en tijdens de vroege fase van infectie. Het verloop van het somatisch celgetal en de schommelingen in het aantal kolonievormende eenheden werden onderzocht voor *E. coli* mastitis. De koeien werden ingedeeld in ‘moderate’ en ‘severe responders’. Daarvoor werden criteria gebruikt die in ons laboratorium reeds eerder werden ontwikkeld.

Voor infectie en tijdens de vroege fase van infectie was de chemiluminescentie van bloed en melk PMN beduidend hoger voor ‘moderate responders’ dan voor ‘severe responders’. Dit is in overeenstemming met hetgeen in ons laboratorium reeds eerder werd aangetoond en stemt overeen met hetgeen thans in de literatuur aanvaard wordt.
Een negatieve correlatie werd gevonden tussen de chemiluminescentie van bloed en melk PMN en de ernst van de klinische symptomen tijdens mastitis. De snelle toename in de chemiluminescentie van melk PMN en in het somatisch celgetal tijdens infectie resulteerde in een verminderd aantal kolonievormende eenheden en in een snel herstel van coliforme mastitis in ‘moderate responders’. Bij de ‘severe responders’ daarentegen was de toename van de chemiluminescentie en het somatisch celgetal onvoldoende om het aantal kolonievormende eenheden in voldoende mate te laten afnemen.

We konden besluiten dat niet alleen de hogere chemiluminescentie maar ook de kinetiek van de chemiluminescentie voor en tijdens *E. coli* infectie ervoor zorgen dat ‘moderate responders’ resistent zijn tegenover *E. coli* infectie. Dit is de eerste keer dat een relatie aangetoond wordt tussen de kinetiek van de chemiluminescentie van bloed en melk PMN en de ernst van het verloop van *E. coli* mastitis. Onderzoek naar het onderliggend biochemisch mechanisme van deze bevindingen zou ons meer inzicht kunnen geven in de complexe fysiopathologie van mastitis bij hoogproductief melkvee tijdens de vroege lactatie. Het is duidelijk dat, aangezien de studiën ook betrekking hebben op onderzoek met bloed PMN de conclusies kunnen geschets worden in een bredere context van puerperale infectie met opportunistische kiemen in het algemeen.
Chakideh
(Persian Summary)
چکیده
هدف اصلی این پژوهش بیان نامه بررسی فعالیت انفجار تنفسی و قدرت بقاء نترافیلهای خون و شیر در زمان‌های قبل و بعد از ایامان و فوتوانسته‌ی میانی‌وارد شکل اصلی بررسی فعالیت نترافیلهای سازی نانوساین آن مخصوصا نترافیلهای شیر میباشد. در خشخاشهای 0.2 پهلوی روش جدانصاری، قابلیت بقاء و تجزیه فعالیت نترافیلهای مورد مطالعه قرار گرفت که نتایج حاصل مورد استفاده روش فلوسیتومتری میباشد.
در dau مطالعه فعالیت نترافیلهای شیر و خون بروز افتاده و سلول‌های شیر و خون بروزتولید می‌شوند.
شاید ارتباط با کنتینگ کولمبینهای شیر و شیر سرگردی شود.
عوامل فیزیولوژیک (دوره شیر دهی و تعداد زایمان) روی کمپوننت‌هایی و قدرت بقاء نترافیلهای خون و شیر در بخش‌های 40 مورد بررسی قرار گرفت و نتیجه حاصل برابر ایجاد نهایی که در این بخش نامه به ظرفیت می‌گردد. مشاهدات میان افزايش شیر میکروسنس نترافیلهای تعیین شد. نسبت به میکروسنس حسین تری گردید. در حالت بقاء نترافیلهای تابع مکعب حسین گردید. دهی در همین مدت کمیکین (Kinetics) نتایج دهی گردید. این نتایج در گروه به روی کمپوننت‌های شیر سرگردی شیر و شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگردی شیر سرگر
یافته، در حالی که قدرت بیع تروفیلیا خون ثابت بود. در طول این دوره پستان با اندوکسین یا E. coli
کمیولیمینسنس و قدرت بیع تروفیلیا کوارترهای آلوده احتمال حدود شدت این بیماری پستان کمتر می‌شود.
در انتهای (یکشنبه 8) تاثیر کمیولیمینسنس تروفیلیا خون و شیر در مرحله قبل و فاز اول عفونت، روی
شدت عفونت برسی شد. تغییرات طول عفونت پستان ناشی از E. coli
نیز بررسی گردید. بر اساس شدت عفونت حاصله، گاوها به دو گروه طبقه بندي گرديد. در گروه Severe (S)، Moderate (M)
کمیولیمینسنس تروفیلیا خون و شیر و شدت عفونت بیشتر از گروه S1 در ترکیب و فاز اول عفونت بود. بین کمیولیمینسنس تروفیلیا خون و شیر و شدت عفونت
پستان ارتباط منفی وجود داشت. افزایش سریع کمیولیمینسنس تروفیلیا شیر و SCC
کاهش سریع عفونت پستان در گروه M شد. ولی افزایش کمیولیمینسنس تروفیلیا و CFU
برای کنترل CFU در گروه M کافی بود. عاملی که باعث افزایش قدرت دفاعی پستان گاوها M
بود، فقط افزایش مطلق کمیولیمینسنس بنده است بلکه کنتیک کمیولیمینسنس در قبل و بعد از عفونت
پستان هم به دست ارتباط بین کنتیک کمیولیمینسنس تروفیلیا خون و شیر و عفونت پستان برابر اولین بار
در این پایان نامه گزارش شده است. کاهش تولید رادیکالهای آزاد داخل سلولی میتواند علت اصلی تضعیف
قدرت گروه به اطراف پوستی به تروفیلیا های شیر در گاوها گیاهی.

مطالعات بیشتر بر روی مکانیسم بوشیمیایی یافته های های مندرج در این پایان نامه میتواند بیان بیان
قدرت تفکیم ما به پیچیدگی پاتوژنیولوژی عفونت پستان و سایر بیماری‌های عفونی مرتبط به آغاز شیر دهی در
گاوها یا پرتولید گردد.
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