

## Apoptosis and necrosis of blood and milk polymorphonuclear leukocytes

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**Abstract** – Increased milk somatic cell counts (SCC) are used as an indicator for bovine mastitis. During mastitis, polymorphonuclear leukocytes (PMN) become the predominant cell type. Shortly after parturition, the severity of mastitis is increased and several PMN functions are downregulated. Apoptotic and necrotic processes of PMN could influence SCC and PMN functions. In this study, the percentages of apoptotic and necrotic PMN in blood and milk from early and midlactating healthy cows were compared. Apoptosis and necrosis of PMN were quantified using a dual-color flow cytometric procedure with fluorescein labeled annexin-V (green) and propidium iodide (red). Using this technique three different subpopulations of bovine PMN could be detected: apoptotic cells (high intensive green fluorescence), necrotic cells (high intensive green and high intensive red fluorescence) and viable cells (low intensive green and low intensive red fluorescence). Following a 4 h incubation of blood from both groups of cows at 37 °C to induce apoptosis, the mean percentage of apoptotic blood PMN was significantly higher ( $P < 0.01$ ) in early lactating cows (15.1%,  $n = 9$ ) compared with midlactating cows (5.3%,  $n = 10$ ). The mean percentage of necrotic PMN remained lower than 5% in all cows. In contrast to blood, no significant difference was found between the percentage of apoptotic PMN in milk from early (41.2%,  $n = 7$ ) and midlactating cows (34.0%,  $n = 8$ ). The percentage of necrotic PMN in milk from early lactating cows (25.9%,  $n = 7$ ) was significantly higher than that in midlactating cows (14.2%,  $n = 8$ ) ( $P < 0.05$ ). Higher percentages of apoptotic as well as necrotic PMN were consistently found in milk compared to blood in all cows. From these results, it can be concluded that spontaneously induced apoptosis was higher in blood PMN from early lactating cows than in blood PMN from midlactating cows. The higher percentage of necrotic milk PMN in early lactating cows than in midlactating cows could be explained by the induction of secondary necrosis.

**bovine neutrophil / apoptosis / necrosis / blood / milk**

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**Résumé – Apoptose et nécrose des polynucléaires neutrophiles du sang et du lait de vaches en début et en milieu de lactation.** Le nombre total de cellules somatiques (SCC) du lait est utilisé pour détecter les mammites. Au cours des mammites, le SCC est augmenté et les polynucléaires neutrophiles (PMN) deviennent le type prédominant des cellules. Peu de temps après la parturition, la gravité des mammites est augmentée et plusieurs fonctions des PMN sont diminuées. Les processus apoptotiques et nécrotiques des PMN pourraient influencer les SCC et les fonctions des PMN. Dans cette étude, les pourcentages des PMN apoptotiques et nécrotiques du sang et du lait des vaches au début de lactation et des vaches en milieu de lactation ont été comparés. L'apoptose et la nécrose des PMN ont été mesurées en utilisant une technique de cytométrie en flux par un procédé de double coloration utilisant l'annexine-V marqué par de la fluorescéine (vert) et de l'iode de propidium (rouge). Par cette technique trois sous-populations différentes de PMN bovins ont pu être détectées: les cellules apoptotiques (intensité élevée de la fluorescence verte), les cellules nécrotiques (intensités élevées des fluorescences rouge et verte) et cellules viables (faible intensité des fluorescences rouge et verte). Le sang des vaches en début de lactation et en milieu de lactation a été incubé *in vitro* pendant 4 h à 37 °C pour induire l'apoptose. Après cette incubation, le pourcentage moyen des PMN apoptotiques du sang était sensiblement plus élevé ( $P < 0,01$ ) chez les vaches en début de lactation (15,1 %,  $n = 9$ ) que chez les vaches en milieu de lactation (5,3 %,  $n = 10$ ). Cependant, le pourcentage moyen des PMN nécrotiques est resté en dessous de 5 %. Contrairement au sang, aucune différence n'a été trouvée entre le pourcentage des PMN apoptotiques du lait des vaches au début de lactation (41,2 %,  $n = 7$ ) et celui des vaches en milieu de lactation (34,0 %,  $n = 8$ ). Le pourcentage des PMN nécrotiques du lait des vaches en début de lactation (25,9 %,  $n = 7$ ) était significativement plus élevé que le pourcentage des PMN nécrotiques des vaches en milieu de lactation (14,2 %,  $n = 8$ ) ( $P < 0,05$ ). De façon générale, les pourcentages des PMN apoptotiques et des PMN nécrotiques étaient plus élevés dans le lait que dans le sang chez toutes les vaches. On peut conclure que l'apoptose spontanée était plus élevée dans les PMN du sang des vaches en début de lactation que dans les PMN du sang des vaches en milieu de lactation. Le pourcentage des PMN nécrotiques plus élevé dans le lait des vaches au début de lactation que dans celui des vaches en milieu de lactation peut être expliqué par l'induction d'une nécrose secondaire.

**neutrophile bovin / apoptose / nécrose / sang / lait**

## 1. INTRODUCTION

In healthy animals, production and removal of polymorphonuclear leukocytes (PMN) is tightly regulated, which keeps their numbers in blood, milk and tissues constant. The life cycle of bovine PMN is brief. In humans, PMN maturation in the bone marrow requires 10 to 14 days [1]. After maturation, PMN may be stored for a few additional days. The 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 briefly in the blood stream (circulating pool, half-life of 8.9 h), attach to the vascular endothelium (marginal pool), leave the blood stream by diapedesis between endothelial cells and mammary epithelium, and enter the mammary gland where they function as phago-

cytes for 1-2 days. PMN form the first line of immunological defence against bacteria invading the bovine mammary gland. Older PMN are removed by macrophages as they undergo apoptosis without inducing an inflammatory response.

Sládek and Ryšánek [16] were the first to show that bovine PMN are phagocytosed by macrophages in the mammary gland of heifers. In contrast, necrosis can lead to an inflammatory reaction because of the release of the cellular content into the surrounding tissue. Apoptotic PMN have decreased cell activity [22]. In the blood stream, apoptotic or necrotic PMN are seldomly observed. Apoptosis occurs specifically in the bone marrow and at the tissue level [3]. However, PMN may be activated in the circulation for an accelerated apoptotic cell death in the tissues. Besides machine milking twice a day, apoptosis of PMN regulates the

clearance of PMN from the inflammatory site, and is thereby an important aspect in controlling the inflammatory response of the bovine mammary gland.

Increased somatic cell count (SCC) of milk is now used as a parameter to determine mastitis. The cells in normal milk consist of lymphocytes, PMN, macrophages and epithelial cells [8]. In infected mammary glands PMN are the predominant cell type. Earlier studies have shown that SCC prior to infection determines the sensitivity and severity of mastitis in lactating cows [4, 21]. The protective effect of a high SCC against mastitis pathogens is well known [15]. Shortly after parturition, several PMN functions are downregulated and the severity of mastitis is increased [2,5]. At present, it is still unclear whether apoptosis could determine the SCC and the defence capacity of the mammary gland. It is most likely that apoptotic processes in the milk could influence cell functions and SCC because apoptotic PMN are removed by macrophages. In this study, the percentages of necrotic and apoptotic PMN in blood and milk were compared in early and midlactating cows.

## 2. MATERIALS AND METHODS

### 2.1. Animals

Clinically healthy Holstein dairy cows in their 2nd to 6th lactation were selected from the Ghent University dairy herd (Bio-centrum Agri-Vet, Melle, Belgium). All cows were free from intramammary infections. Mean milk somatic cell count from each quarters were determined to be < 100,000 cells/mL. For the flow cytometric analysis, blood and composite milk samples of two groups of lactating cows were taken: 9 early lactating cows ( $13 \pm 6$  days after parturition) and 10 midlactating cows ( $170 \pm 26$  days after parturition).

### 2.2. Blood sampling

Blood was collected from the jugular vein by venipuncture in vacutainer tubes containing 125 I.U. heparin as anticoagulant (Laboratoire EGA, Nogent-Le-Roi, France). Smears were prepared from whole blood and stained with Hemacolor (Merck, Darmstadt, Germany). Differential microscopic counts were determined by counting 100 cells. During the selection of the animals, cows with eosinophil counts greater than 10% were excluded.

### 2.3. Apoptosis and necrosis in blood PMN

As apoptosis does not occur in circulation, 100  $\mu$ L blood from early and midlactating cows was incubated in vitro for 4 h at 37 °C to induce apoptosis. PMN were labeled with fluorescein isothiocyanate (FITC)-annexin-V (dilution 1/100; Boehringer Mannheim GmbH, Mannheim, Germany) and cellular DNA was stained using propidium iodide (PI) (final concentration of 1  $\mu$ g/mL, Sigma Chemical Co., St Louis, MO, USA) in the presence of  $\text{Ca}^{2+}$  rich medium (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM  $\text{CaCl}_2$ ) as previously described [19]. Erythrocytes were lysed with 600  $\mu$ L formic acid (1.4 mg/mL). The remaining leukocytes were stabilized with 265  $\mu$ L of buffer (6.0 g sodium carbonate, 14.5 g sodium chloride, 31.3 g sodium sulphate/L). Apoptosis and necrosis was then determined according to Van Oostveldt et al. [19].

### 2.4. Apoptosis and necrosis in milk PMN

Apoptosis of milk cells was quantified on freshly isolated cells without prior induction of apoptosis. Milk samples (1.5 mL) were added to 3.5 mL phosphate buffered saline (PBS) and centrifuged during 10 min

at  $180 \times g$ . The fat was removed, the supernatant was discarded and the tubes were kept upside down until they were carefully cleaned with a cotton swab to remove fat adhering to the test tube wall. The pellet was resuspended in 50  $\mu\text{L}$  PBS. PMN were labeled by adding 50  $\mu\text{L}$  of FITC-annexin-V (dilution 1/50) and PI (final concentration of 1  $\mu\text{g}/\text{mL}$ ) in the presence of  $\text{Ca}^{2+}$  rich medium as described above.

## 2.5. Flow cytometry

A FACScan flow cytometer (Becton Dickinson, Immunocytometry Systems, San José, CA, USA) was used in all the experiments. Linear amplification of the forward scatter (FS) and side scatter (SS) light signals was set with logarithmic amplification of the fluorescence signals. The 488 nm excitation wavelength was used. For each sample, at least 1000 PMN were acquired. PMN were selected for analysis by gating on the FS and SS dot plot. FITC and PI fluorescence was measured through 530/30 and 585/42 band pass filters, respectively. Compensation for FITC – % PI and PI – % FITC was 2.0 and 36.2%, respectively. Data were acquired and processed using CELL Quest software (Becton Dickinson).

## 2.6. Statistical analysis

All statistical procedures, means, standard deviations and standard errors of the means were computed using statistical software (Statistix<sup>®</sup> NH Analytical Software, Tallahassee, FL, USA) according to Snedecor and Cochran [18]. Results are expressed as means plus or minus standard error of the means. Normality was tested using the Wilk-Shapiro test. Statistical analysis was carried out using the Kruskal Wallis nonparametric test for pairwise comparison of means with cows as the randomized factor. Statistical significance was accepted at  $P < 0.05$ ,  $P < 0.01$  and  $P < 0.001$ .

## 3. RESULTS

### 3.1. Apoptosis and necrosis in blood PMN

Following a 4 h incubation of blood, no differences in the percentage of necrotic blood PMN of early (3.2%) and midlactating cows (4.3%) were observed. The mean percentage of apoptotic blood PMN was 15.1% in early lactating cows compared to 5.3% in midlactating cows. A significant difference between both groups was observed ( $P < 0.01$ ) (Tab. I).

**Table I.** Percentages of apoptotic and necrotic PMN in blood following a 4h in vitro incubation at 37 °C and milk of early and midlactating cows. Data are means  $\pm$  SEM.

	Early lactation	Midlactation	Statistical significance
Apoptotic PMN (%)			
Blood	15.1 $\pm$ 3.0	5.3 $\pm$ 1.1	$P < 0.01$
Milk	41.2 $\pm$ 5.4	34.0 $\pm$ 2.0	NS*
Necrotic PMN (%)			
Blood	3.2 $\pm$ 1.0	4.3 $\pm$ 1.5	NS*
Milk	25.9 $\pm$ 5.9	14.2 $\pm$ 3.2	$P < 0.05$

\* NS = not significant.

### 3.2. Apoptosis and necrosis in milk PMN

A higher percentage of necrotic and apoptotic PMN could be detected in milk than in blood. The percentage of necrotic PMN in milk from early lactating cows (25.9%) was significantly ( $P < 0.05$ ) higher than the percentage of necrotic PMN from midlactating cows (14.2%). The percentage of apoptotic PMN in milk was significantly higher compared to blood. However, there was no difference between the percentage of apoptotic PMN in milk from early lactating cows (41.2%) and midlactating cows (34.0%) (Tab. I).

## 4. DISCUSSION

Apoptosis is characterized by typical morphological features. In this study, the expression of the phospholipid phosphatidyl serine on the outer membrane of blood and milk PMN was determined with annexin-V as one of the early features of apoptosis. Earlier studies have confirmed that phosphatidyl serine expression on bovine PMN is correlated with other morphological features of apoptosis such as chromatin condensation and a picnotic nucleus (Van Oostveldt et al., unpublished results).

Spontaneously induced apoptosis was higher in blood PMN from early lactating cows than in blood PMN from midlactating cows. This suggests that blood PMN shortly after parturition are more activated for apoptosis. Apoptotic PMN have decreased functions [22]. Increased PMN apoptosis could therefore explain the decreased blood PMN functions, such as diapedesis and respiratory burst activity, shortly after parturition.

We observed a much higher percentage of apoptotic PMN in milk than in circulation. Diapedesis of blood PMN through the blood-milk barrier induced PMN apoptosis [20]. Apoptotic processes in the mammary gland could explain the fact that PMN func-

tions of milk PMN are decreased compared to blood PMN. Many previous studies have shown that the functions of milk PMN are decreased compared to blood PMN [6-8,10-11]. In vitro diapedesis of bovine PMN through a monolayer of isolated mammary gland epithelial cells induced a decreased phagocytosis and respiratory burst capacity of PMN [17]. Apoptosis of PMN following diapedesis could explain this phenomenon. Indeed, Whyte et al. [22] have shown that human apoptotic PMN have decreased cell functions. Cell spreading, chemotaxis, phagocytosis, production of myeloperoxidase and the release of superoxide radicals following zymosan stimulation are decreased in a PMN population with an increased percentage of apoptotic PMN [22]. Our findings therefore suggest that apoptosis is induced by in vivo PMN migration into the mammary gland, thereby contributing to a decreased milk cell function.

The percentage of apoptotic PMN in milk was not significantly different between early and midlactating cows. This might be due to the fact that a part of the apoptotic PMN that have ingested fat globules might be removed during the isolation procedure together with the fat layer following the centrifugation of the milk samples. However, the removal of milk fat is generally accepted to be essential before flow cytometric analysis of milk cells [9,12]. The percentage of necrotic PMN in milk was significantly higher in early lactating cows. A possible explanation is that necrosis of milk PMN is in fact a secondary necrosis following apoptosis. If apoptotic cells are not phagocytosed by macrophages they eventually die through secondary necrosis [13,14]. This could explain why different levels of spontaneously induced apoptotic blood PMN in both groups were not reflected in different levels of apoptotic milk PMN.

In conclusion, spontaneously induced apoptosis was higher in blood PMN from early lactating cows than in blood PMN from midlactating cows. Whereas apoptosis of milk PMN was similar, the percentage

of necrotic milk PMN was higher in early lactating cows than in midlactating cows. This phenomenon could be explained by the induction of secondary necrosis. Our findings suggest that increased percentages of apoptotic blood PMN and necrotic milk PMN in early lactating cows may be a possible risk factor for severe mastitis shortly after parturition.

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