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APOPTOSIS OF NEUTROPHILS, MONOCYTES, AND LYMPHOCYTES IN THE PERIPHERAL BLOOD OF COWS DURING LACTATION

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Abstract

The immune system of animals plays an important role in the homeostasis system. The aim of the study was to detect apoptosis of immune cells in the peripheral blood stream at different periods of lactation in cows. The investigation of the immune defense of cows was conducted during various lactation periods: early (3–5 days), mid (90–150 days), and late (drying off – 5–7 days; dry period – 12–20 days) periods of mammary gland function. The highest intensity of apoptosis was observed in neutrophils and monocytes during the mid-lactation period. The highest intensity of apoptosis is observed in phagocytic cells during the colostrum secretion period and mammary gland involution. The study of physiological constants of blood cell apoptosis will serve as a basis for developing informative methods of mastitis diagnosis in cows and implementing effective treatment measures. The findings obtained from this research are valuable for practical veterinarians and from a public health perspective.

Introduction

Currently, there is active research worldwide on measures for preventing cow mastitis. It is important to emphasize that some researchers draw parallels between non-specific immunobiological reactivity and resistance, constantly exploring innovative ways of molecularly regulating immune response, including apoptosis of immune cells. This includes investigating the role of various immunomodulators, such as cytokines, growth factors, and signaling molecules, in enhancing the immune system's ability to resist mastitis-causing pathogens (LIU 2018, CVETNIC et al. 2021). Researchers are exploring novel vaccination strategies, probiotics, and nutraceuticals that can enhance the cow's natural defenses against

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mastitis. These efforts underscore the ongoing commitment to developing effective and sustainable methods for preventing this prevalent disease in dairy cattle (Burton et al. 2003, Lim et al. 2017, Zhelavskyi 2021).

The process of apoptosis is the outcome of diverse factors that contribute to cell death. These factors may encompass nonspecific elements like temperature fluctuations, exposure to toxic substances, oxidants, free radicals, as well as γ and ultraviolet radiation, along with bacterial toxins. In all of these cases, apoptosis is induced, but with increasing impact of the respective agent, necrotic cell death is initiated (Wang et al. 2019, Cvetnic et al. 2021), which has sparked increased interest among researchers, particularly in hormonal regulation of apoptosis (Wynn and Vannella 2016, Kydonaki et al. 2021, Wang et al. 2020, Zhelavskyi et al. 2021).

The initiation of apoptosis is caused by physiological signal-inducing molecules that are recognized by specialized cellular receptors, triggering a cascade of subsequent intracellular biochemical processes (Zhou et al. 2015, Bae et al. 2022). These signals can originate from various factors, including biologically active substances, hormonal imbalances, antigen overload, the presence of specific antibodies to cell receptors, cytokines, and others (Psaila et al. 2016, Schnabel et al. 2020). Apoptosis can be prevented or regulated by various factors, including biological molecules that promote or inhibit apoptosis, as well as regulatory intracellular mechanisms (Levin et al. 2016, Kim et al. 2021, Zhelavskyi et al. 2024). Considering that apoptosis is a general biological mechanism of regulation and balance, responsible for maintaining the physiological balance of cell populations and eliminating distorted, mutated, and defective cells, new approaches are being considered for the treatment and prevention of diseases.

Therefore, this study aimed to determine the process of physiological aging and cell death in the peripheral bloodstream at different stages of lactation in cows.

Materials and Methods

Animals' criteria. A total of 112 cows (*Bos Taurus*, Ukrainian black-and-white milk breeding) ranging from 3 to 5 years of age were selected for an experiment. Cows were divided into four groups. Each group consists of 28 animals. The first group cows in the period of colostrum secretion (3–5 days); the second – cows in the middle lactation period (90–150 days); the third – drying off (5–7th day) and the fourth group – in the dry period (12–20th day). All animals were clinically healthy and belonged to the

farms of the Khmelnytskyi and Vinnytsia regions of Ukraine. Blood samples were taken from animals between 7:00 and 9:00 in the morning. Blood was taken from *v. jugularis* into vacuum-sealed glass containers.

This investigation was approved according to the Law of Ukraine "On the Protection of Animals from Cruel Treatment" (No. 3447-IV of February 21, 2006) and according to the requirements of the European Convention for the Protection of Pet Animals (ETS No. 125, Strasbourg, 13/11/1987). All experiments were carried out with the Ethical Permit at the Vinnytsia National Agrarian University, Ukraine. All animal manipulations were performed by the European Convention for the Protection of Vertebrate Animals and used for experimental and scientific purposes (Strasbourg, 18 March 1986).

Apoptosis analysis and detection

A blood sample was collected into a tube containing an anticoagulant Ethylenediaminetetraacetic acid (EDTA, Maxwell®, USA), and it was mixed gently to prevent clotting. The volume of Lymphoprep needed was calculated based on the amount of blood and desired cell yield, ratio of 1:1 (volume of blood to volume of Lymphoprep, Axis Shield Poc AS, Oslo, Norway) was used. The calculated volume of Lymphoprep was layered at the bottom of a centrifuge tube using a pipette. The whole blood sample was carefully layered on top of the Lymphoprep solution using a slow and gentle pouring technique to avoid disturbing the layers. The tube was centrifuged at specific settings optimized for cell separation. The recommended conditions for most blood cell isolations were: centrifugation at 800–1000 x g for 20–30 minutes at room temperature (20–25°C) without braking. A swing-out rotor was used if available to prevent mixing of layers during centrifugation. After centrifugation, three distinct layers were visible in the tube: the top layer containing plasma and lymphocytes, the middle layer containing monocytes, and the bottom layer containing granulocytes, including neutrophils.

Each layer was carefully aspirated and transferred into separate tubes using a Pasteur pipette or a pipetting device. The top layer (lymphocytes) was transferred into one tube, the middle layer (monocytes) into another tube, and the bottom layer (neutrophils and granulocytes) into a third tube. Each cell population was washed with Phosphate-buffered saline (PBS, pH 7.4; Sigma-Aldrich, USA) or a suitable buffer to remove Lymphoprep and other contaminants. The cells were then centrifuged at a low speed (300 x g) for 10 minutes to pellet the cells. The supernatant was discarded, and the cell pellets were resuspended in the desired medium or buffer for further analysis or experiments. Quality control and viability

assessment were performed by cell counting and viability assessment using a automated cell counter Nexcelom Cellometer Auto T4 (Nexcelom Bioscience, USA).

Cells were washed with PBS to remove any residual media or serum. The cell concentration ($5 \cdot 10^6$ cells/mL). The cells were then resuspended in 1X binding buffer provided with the Annexin V-FITC/PI staining kit (PharMingen, Becton Dickinson, USA). Annexin V-FITC was added to the cell suspension. The cells were incubated in the dark at room temperature (18°C) for a specified time (25 minutes) to allow Annexin V-FITC binding. After Annexin V-FITC incubation, propidium iodide (PI; PharMingen, Becton Dickinson, San Diego, CA, USA) was added to the cell suspension (5 µg/mL). The cells were incubated for an additional 5 minutes in the dark at room temperature (18°C) to allow PI staining. Following staining, the cells were analyzed using a flow cytometer equipped with appropriate filters for FITC (green) and PI (red) fluorescence. Compensation controls and gating strategies were set up to distinguish between live (Annexin V-FITC negative, PI negative), early apoptotic (Annexin V-FITC positive, PI negative), late apoptotic/necrotic (Annexin V-FITC positive, PI positive), and necrotic (Annexin V-FITC negative, PI positive) cell populations. Flow cytometry data was acquired and analyzed using software such as FlowJo or BD FACSDiva to quantify the percentages of different cell populations based on their Annexin V-FITC and PI staining patterns. The flow cytometry data was interpreted to determine the extent of apoptosis and necrosis in the cell population. Apoptotic indices, such as the ratio of early apoptotic cells to total cells (early + late apoptotic), were calculated to assess apoptosis induction.

Statistical analysis

The values in this investigation are presented as mean ±SD. Data were analyzed by one-way analysis of variance (MANOVA). Differences were considered statistically significant at a *P*-value of less than 0.05 (Statistica® 12.6, StatSoft, USA).

Results

The beginning of lactogenesis was characterized by a certain activation of the apoptotic process in the phagocytic defense system: the relative (14.54 ±0.52%) and absolute quantity ($103.3 \pm 7.57 \cdot 10^9$ cells/µL) of neutrophils with signs of apoptosis increased in the peripheral bloodstream (Table 1).

Table 1

Changes in cell apoptosis in peripheral blood of cows during different lactation periods

Lactation periods	The intensity of spontaneous apoptosis (ISA) [%]						CAB (L : M : N)
	lymphocytes		monocytes		neutrophils		
	%	· 10^9 cells/μL	%	· 10^9 cells/μL	%	· 10^9 cells/μL	
Colostrum period (n = 28)	3.72 ±0.46	26.37 ±1.45	1.8 ±0.40	7.73 ±0.34	14.5 ±0.52	103.3 ±7.57	0.23 ±0.06
Mid-lactation period (n = 28)	4.57 ±0.50*	35.4 ±1.71**	0.53 ±0.05**	4.12 ±0.45**	5.07 ±0.26**	39.3 ±1.52**	0.89 ±0.10
Drying off (n = 28)	5.16 ±0.30**	37.99 ±2.15**	0.79 ±0.04*	8.07 ±0.75*	12.08 ±0.40*	91.14 ±2.14*	0.43 ±0.03
Dry period (n = 28)	5.36 ±0.49**	36.88 ±2.35**	1.18 ±0.39	8.28 ±0.83**	15.27 ±0.45*	107.6 ±5.54*	0.31 ±0.07**

Explanations: * – $P < 0.05$; ** – $P < 0.01$ – regarding indicators at the beginning of lactation; L – lymphocytes; M – monocytes; N – neutrophils; CAB – Cell Apoptosis Balance

The apoptosis index of monocytes, in this case, was 1.8 ±0.40%, which is also associated with the direct involvement of these cells in the morphology and functional restructuring of the mammary gland parenchyma, as well as the active elimination of dead neutrophils. During the colostrum secretion period, the apoptosis index of lymphocytes was the lowest, amounting to 3.72 ±0.46%, which was also reflected in the lowest quantitative value (26.37 ±1.45 · 10^9 cells/μL). In the early lactation period, there was also a certain redistribution of individual populations of cells, which was reflected in the high value of the lymphocyte-to-monocyte-to-neutrophil (L : M : N) population ratio (0.23 ±0.06) and the monocyte-to-neutrophil (M : N) ratio (0.08), informative indicators of phagocytic cell apoptosis predominance (Figure 1). In the middle period of lactation, changes in the intensity of cell apoptosis primarily affected neutrophils in peripheral blood. As a result, the spontaneous apoptosis index of microphage cells decreased by 9.47% ($P < 0.01$), which was reflected in their absolute quantity (39.3 ±1.52 · 10^9 cells/μL), compared to the initial value of 103.3 ±7.57 · 10^9 cells/μL. Simultaneously, the intensity of spontaneous physiological death of monocytes decreased, while the spontaneous apoptosis of lymphocytes slightly increased and amounted to 4.57 ±0.50% (35.4 ±1.71 · 10^9 cells/μL). In the post-colostrum period, lymphocytes also undergo changes in their functional activity. The apoptosis of the investigated immune-competent cells remained within the range of physiological constants, as indicated by the changing parameters L : M : N (0.89 ±0.10) and M : N (0.10 ±0.01) ratios.

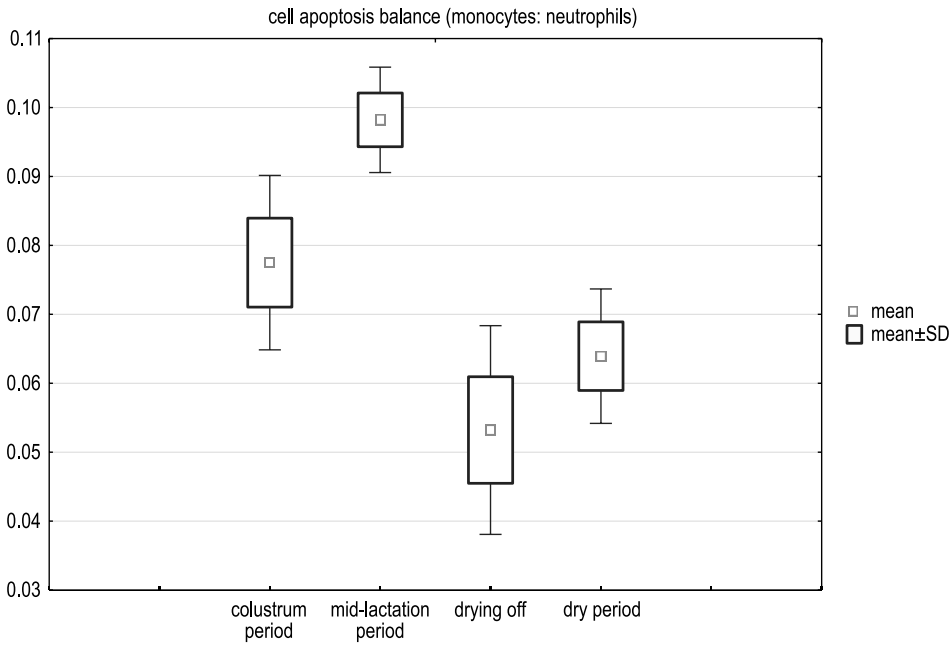

Fig. 1. The balance of apoptosis of phagocytes (Monocytes: Neutrophils) of cows in different periods of lactation

The initial involution processes in the mammary gland, which began during the drying-off period, continued into the dry period and were reflected in the process of spontaneous apoptosis of the investigated blood cells. The proportion of apoptotic neutrophil granulocytes observed during this time was at the level of 15.27 ±0.45% (107.6 ±5.54 · 10^9 cells/μL). There was also an increase in the proportion of monocytes that completed their life cycle (1.18 ±0.39%, 8.28 ±0.83 · 10^9 cells/μL). The population ratio in the L : M : N formula during the drying-off period was 0.43 ±0.03 and 0.31 ±0.07, indicating a cellular redistribution of immune competent cells towards the activation of spontaneous apoptosis in phagocytic cells, which directly participate in the elimination of metabolites expelled from the cow's mammary gland.

Discussion

Apoptosis, also known as programmed cell death, plays a crucial role in maintaining the balance and functionality of the immune system. This process is vital for various aspects of immune function, including the elimination of damaged or infected cells, regulation of immune responses, and

development of immune cells (PSAILA et al. 2016, LI et al. 2022). One key importance of apoptosis in the immune system is its role in eliminating cells that are no longer needed or have become harmful. For example, during an immune response, activated immune cells such as T-cells and B-cells undergo apoptosis after they have completed their tasks (LEVIN et al. 2016, KYDONAKI et al. 2021). This helps prevent excessive immune activation and inflammation, which can be detrimental to the body (HEISER et al. 2018, ZHELAVSKYI et al. 2021). Apoptosis is essential for removing self-reactive immune cells, which can cause autoimmune diseases if left unchecked (THEURL et al. 2016). Through apoptosis, immune cells that mistakenly target the body's own tissues are eliminated, contributing to immune tolerance and preventing autoimmune reactions. Apoptosis is involved in shaping the immune cell repertoire during development (SCHNABEL et al. 2020, SONG et al. 2022). Apoptosis plays a role in immune cell homeostasis, helping to maintain a balance between different immune cell populations (ZHELAVSKYI et al. 2023a). Excessive cell proliferation or survival can lead to immune disorders, and apoptosis helps regulate the numbers of immune cells to ensure optimal immune function (SUN et al. 2018, LIU 2020, ZHELAVSKYI et al. 2023b).

There is published data that various factors can affect the process of apoptosis of the blood cells of cows, including the inhibited level of the inflammatory factor in the peripheral circulation. In particular, there is information that apoptosis can be induced under the influence of excessive concentrated feeding of animals (ZHOU et al. 2024). In this study, we examined the mechanisms that maintain immune homeostasis during different stages of lactation. We confirmed that in different periods of lactation in the peripheral blood of cows, dynamic changes occur in the cell population with the manifestation of apoptosis. This discovery opens a new way to diagnose bovine mastitis and monitor the effectiveness of treatment and provides insight into how to perform effective immunomodulation.

During the early lactation period, a portion of neutrophil granulocytes, which had fulfilled their function, also underwent apoptosis, confirming our initial assertion made at the beginning of the experimental series. The issue of apoptosis in immune-competent cells of the mammary gland has attracted the attention of other researchers as well (ZHOU et al. 2015, BAE et al. 2022).

Bacterial components such as lipopolysaccharides can activate the immune defense of the mammary gland, leading to the recruitment of immune cells like neutrophils, macrophages, and lymphocytes to the site of inflammation. Mammary epithelial cells not only serve as a barrier but also respond to antigens by producing an inflammatory response. Intersti-

tial fibroblasts also play a role in the inflammatory process (XU et al. 2021). When the mammary gland faces bacterial invasion, macrophages in both breast tissue and milk recognize the invading pathogens, triggering an inflammatory response (SUN et al. 2018, LIU 2020). They also release pro-inflammatory substances to attract neutrophils to the area of inflammation within the mammary gland, aiding in bacterial resistance. Healthy mammary gland cells in breast tissue and milk primarily consist of breast epithelial cells and macrophages, whereas diseased tissues and their secretions predominantly contain transformed macrophages that become neutrophils (ANWER et al. 2016, ZHELAVSKYI et al. 2023a). One of the key functions of apoptosis in mastitis is its involvement in the removal of damaged or infected cells from the mammary gland. When bacteria or other pathogens penetrate the udder and cause inflammation, mammary gland cells may initiate apoptosis to prevent the spread of infection and tissue damage. During mastitis, the immune system is activated, but excessive inflammation can be harmful to the mammary tissues. Apoptosis helps limit this inflammatory response by reducing the number of activated immune cells and restoring balance in the udder. Some studies indicate that imbalanced apoptotic activity may be associated with the development of mastitis. If apoptotic removal of damaged cells is not effective, it can lead to worsened inflammation and infection (AKARAPHUTIPORN et al. 2021, KHAN et al. 2024). Therefore, understanding and controlling the apoptotic process in mastitis are crucial aspects for improving treatment and preventing this disease in cows.

Conclusion

The cow's lactation is characterized by dynamic changes in the manifestation of the apoptotic process of immune cells in peripheral blood. The highest intensity of apoptosis is observed in phagocytic cells (neutrophils and monocytes) during the colostrum secretion period and mammary gland involution. Lymphocyte apoptosis remains within the limits of physiological homeostasis throughout the study. Studying the physiological constants of blood cell apoptosis will serve as a basis for developing informative methods for diagnosing mastitis in cows and implementing effective treatment measures. The findings obtained from this research are valuable for practical veterinarians and from a public health perspective.

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