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Ontogenetic features of the formation of local immune protection of the mammary gland of cows (literature review and original research)

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The authors present modern scientific data on the local immune protection of the mammary gland of cows. Main stages of ontogenetic development of cellular immunity of the mammary gland of cows were traced during clinical and experimental studies. The number of somatic cells in the secret of the mammary gland of the primates was dependent on the period of the functioning of the mammary gland. In the cytology of colostrum mostly ($56.00 \pm 1.90\%$) neutrophil granulocytes were predominant, in the middle period of lactation (3–5th month) the proportion of epithelial cells increased (from 29.51 ± 2.17 to $49.59 \pm 1.94\%$), during the launch period, the population of polymorphonuclear neutrophil granulocytes was changing as well, which virtually recovered to the original level and increased during the dry period. The cytochemical reactivity of intracellular lysozyme of phagocytic cells in the secretion of the breast of the primates was from the beginning of lactogenesis and in the middle period of lactation (3–5th month) almost constant.

However, at the end of lactation, during the onset and dry, with the development of involutionary processes in the mammary gland, a sharp decrease in cytochemical reactivity of intracellular lysozyme of phagocytic cells was observed. A similar trend was observed in the phagosomal activity of lysosomal cationic proteins. The greatest reactivity of phagocytes was shown in the beginning of lactogenesis in reaction to lysosomal cationic proteins, which acquired its maximum manifestation in the middle period of lactation and gradually decreased at the end of the lactation period. In the period of launch and dry in the secretion of pricking cessation, there was a cytochemical activation of oxygen-dependent factors of protection of phagocytic cells.

Consequently, the formation of cellular immune defense takes place in the process of ontogenetic development of the mammary gland of cows. In the firstborn, simultaneously with the formation of secretory function of the mammary gland, there is a gradual formation of natural factors protecting the body. Oxygen-independent and Oxygen-dependent factors of phagocytic protection of the breast of the firstborn are not sufficiently formed, their activation is started from the colostrum period and undergoes a physiological fluctuation throughout the lactation period.

Key words: cows, mammary gland, lactation, local immunity, cellular and humoral protection factors, immunocompetent cells.

Онтогенетичні особливості формування локального імунного захисту молочної залози корів (огляд літератури та оригінальні дослідження)

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В роботі авторами наведено сучасні наукові дані про локальний імунний захист молочної залози корів. Клініко-експериментальними дослідженнями простежено основні етапи онтогенетичного становлення клітинного імунітету молочної залози корів. Кількість соматичних клітин у секреті молочної залози первісток залежала від періоду функціонування молочної залози. В цитограмі молозива в основному переважували ($56,00 \pm 1,90\%$) нейтрофільні гранулоцити, в серед-

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ній період лактації (3–5-й місяць) зростала частка епітеліоцитів (з $29,51 \pm 2,17$ до $49,59 \pm 1,94\%$), в період запуску також змінювалась популяція поліморфоядерних нейтрофільних гранулоцитів, що практично відновлювалась до вихідного рівня і збільшувалася в період сухостою.

Цитохімічна реактивність інтралейкоцитарного лізоциму фагоцитарних клітин секрету молочної залози первісток з початку лактогенезу та в середній період лактації (3–5-й місяць) знаходилась майже на сталому рівні. Проте, наприкінці лактації, в період запуску та сухостою, із розвитком інволютивних процесів у молочній залозі, відзначалось різке зниження цитохімічної реактивності інтралейкоцитарного лізоциму фагоцитарних клітин. Аналогічну тенденцію прослідковано і в фагосомальній активності лізосомальних катіонних білків. Найбільшу реактивність фагоцити проявили на початку лактогенезу в реакції на лізосомальні катіонні білки, що набувало свого максимального прояву в середній період лактації та поступово знижувалось наприкінці лактаційного періоду. В період запуску та сухостою в секреті вим'я первісток відбувалась цитохімічна активація Оксигензалежних факторів захисту фагоцитарних клітин.

Отже, в процесі онтогенетичного розвитку молочної залози корів відбувається становлення її клітинного імунного захисту. У первісток, паралельно із становленням секреторної функції молочної залози, відбувається поступове формування природніх факторів захисту органу. Оксигеннезалежні та Оксигензалежні фактори фагоцитарного захисту молочної залози первісток не достатньо сформовані, їх активація започатковується з молозивного періоду і зазнає фізіологічного коливання впродовж всього періоду лактації.

Ключові слова: корови, молочна залоза, лактація, локальний імунітет, клітинні та гуморальні фактори захисту, імункомпетентні клітини.

Онтогенетические особенности формирования локальной иммунной защиты молочной железы коров (обзор литературы и оригинальные исследования)

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В работе авторами приведены современные научные данные о локальной иммунной защиты молочной железы коров. Клинико-экспериментальными исследованиями прослежены основные этапы онтогенетического становления клеточного иммунитета молочной железы коров первого года лактации. Количество соматических клеток в секрете молочной железы коров зависело от периода функционирования молочной железы. В цитограмме молозива в основном преобладали ($56,00 \pm 1,90\%$) нейтрофильные гранулоциты, в средний период лактации (3–5-й месяц) росла доля эпителиоцитов (от $29,51 \pm 2,17$ до $49,59 \pm 1,94\%$), в период запуска также изменялась популяция полиморфоядерных нейтрофилов, что практически восстанавливалась до исходного уровня и увеличивалась в период сухостою.

Цитохимическая реактивность интралейкоцитарного лизоцима фагоцитарных клеток секрета молочной железы коров с начала лактогенеза и в средний период лактации (3–5-й месяц) находилась почти на постоянном уровне. Однако, в конце лактации, в период запуска и сухостою, с развитием инволюционных процессов в молочной железе, отмечалось резкое снижение цитохимической реактивности интралейкоцитарного лизоцима фагоцитарных клеток. Аналогичную тенденцию прослежены и в фагосомальной активности лизосомальных катионных белков. Наибольшую реактивность фагоциты проявили в начале лактогенеза в реакции на лизосомальные катионные белки, приобретало своего максимального проявления в средний период лактации и постепенно снижалось в конце лактационного периода. В период запуска и сухостою в секрете вымя коров происходила цитохимическая активация Оксигензалежних факторов защиты фагоцитарных клеток.

Таким образом, в процессе онтогенетического развития молочной железы коров происходит становление ее клеточно-го иммунного защиты. У коров первого года лактации происходит постепенное формирование естественных факторов защиты органа, что проходит параллельно со становлением секреторной функции молочной железы. Кислороднезависимые и кислородзависимые факторы фагоцитарного защиты молочной железы при этом недостаточно сформированы, их активация начинается с молозивный периода и испытывает физиологического колебания в течение всего периода лактации.

Ключевые слова: коровы, молочная железа, лактация, локальный иммунитет, клеточные и гуморальные факторы защиты, иммунокомпетентные клетки.

Introduction

The main contemporary issues of evolutionary immunology are primarily related to the study of the ability of an organism to specific antigenic recognition (ie, the appearance of recognition receptor antigen as molecular recognition factors) and to determine the evolutionary origin of lymphocytes. An open question is also the question of changes in immunological responses in an individual's organism in the ontogenesis process (Dranik et al., 2006; Tizard, 2013).

Mastitis of cows is one of the most common pathologies in dairy cattle breeding, which causes significant economic losses. Currently, scientists in many countries of the world constantly search for effective methods of diagnosis, prevention and treatment, but this pathology is still an urgent problem that requires new approaches to its solution (Kessel et al., 2008; Dorland et al., 2009; Yablonskij and Zhelavskij, 2013).

In the 70s of the last century a new scientific direction – reproductive immunology – was created that integrated fundamental and applied immunological studies of related branches of biology and medicine. A new scientific

school of animal reproduction immunology, headed by the doctor of biological sciences, professor, correspondent member of the National Academy of Sciences of Ukraine V.A. Yablonsky, was formed on the territory of Ukraine. Modern researches of this school have greatly expanded the knowledge about the role of immune mechanisms associated with the reproductive capacity of animals. Nowadays, mammalian immunology laboratories have been conducting research on immunology of lactation and improving methods for assessing the immune status of animals (Griesbeck-Zilch et al., 2008; Rains and Jain, 2011; Yablonskij and Zhelavskij, 2014).

Taking into account that the issue of study of immunobiological reactivity and local immune defense of the mammary gland is the subject of a meticulous study of foreign and domestic scientists, we decided to conduct a thorough review of modern scientific literature and to conduct an analysis of the results of our own scientific ontogenesis of immune mechanisms for the protection of the local protection of the mammary gland of cows (Prado et al., 2011; Oudessa and Almeida, 2011).

Material and methods

The research was carried out on cows of Ukrainian black-and-white milk breeding at the farms of the Khmelnytsky region and in the specialized laboratory of immunology of reproduction of mammals of the Faculty of Veterinary Medicine of the Podilsky State Agrarian and Technical University. At the initial stage, the ontogenetic features of the onset of local immunity of the breast of primary cows were studied.

For the study, four groups of analogues (27 animals each) of experimental animals were formed in which the immunological methods determined the status of cellular factors of immune defense of the mammary gland during lactation periods: the first group ($n = 17$) – cows during the secretion of colostrum (3-5th day); second ($n = 32$) – cows in the middle (3-5th month) of the lactation period; the third ($n = 28$) – during the start (5-7th day) and the fourth group ($n = 28$) – during the dry period (12–20 th day).

The immune status of the cows was determined using the developed immunocard (Yablonsky and Zhelavsky, 2014), which included a step-by-step determination of indices of nonspecific resistance and specific immunobiological reactivity. Immunological studies examined the cellular, humoral, and non-specific localized immune secretion of the mammary gland. Phagocytic activity of leukocytes (FA) was determined in reaction with inert polystyrene particles of latex, phagocytic number (FN), phagocytic index (FI), and phagocytic capacity (FC).

During the cytochemical study of phagocytic reactivity, the state of the non-oxygen dependent factors of phagocytic defense was determined, namely, the activity of myeloperoxidase (MPO) and the reactivity of phagocytic cells in the metabolic reaction with nitrosin tetrazolium (NST-test). Oxygen-independent mechanisms of cell defense were determined by the activity of lysosomal cationic proteins (LCP) and intralleukocytic lysozyme (ILL). Determination of the cytochemical

reactivity of phagocytes in the secretion of the mammary gland was carried out according to our patented method (Patent of Ukraine for Utility Model No. 73635, MPK7A61V 10/02 (2006.01), Method for evaluating the antimicrobial reactivity of the cow's breast secretion secretory neutrophils). All studies were conducted in accordance with the Law of Ukraine «On Protection of Animals from Cruel Treatment» (No. 3447-IV of February 21, 2006) and the current requirements of the European Commission for treating vertebrate animals and protecting them from thirst, hunger, malnutrition, discomfort, fear, pain and illnesses.

In the statistical processing of data arrays, the mean arithmetic, mean arithmetic error, Student t-test was determined. Biometric analysis and biometric interpretation of the obtained results were performed using statistical software Statistica v. 10.

Results and discussion

As it is known in plasma and serum of blood mammals, regardless of antigenic stimulation, there is always a complement that is essentially a whole system of 11 different protein components. Under physiological conditions in the secretion of the mammary gland there are low concentrations of complement. In the secretion of the breast of the cows, the concentration of the component of complement C_3 is only 2.5% of its content in the peripheral blood. In the process of inflammation, the exudative reaction is accompanied by the activation of the $C3b/C3bi$ components. The value of $C5a$, which stimulates chemotaxis and migration of neutrophils in the inflammatory site, has been carefully studied. In serological studies in vitro and in vivo, the development of a mastitis for the participation of *Escherichia coli* or *S. uberis*, accompanied by an intensive leukocyte response, was confirmed (Vernay et al., 2012; Zarrin et al., 2014).

The expressed antimicrobial properties also have lactoferrin, which in its structure is Ferum-binding glycoprotein, which is contained in large numbers in the secondary granules of neutrophil granulocytes. According to, the concentration of lactoferrin in the secretion of the mammary gland ranges from 20–200 mg/ml and significantly increases during the inflammation. The formation of free radicals thus causes the destruction of membrane structures and the death of microorganisms. Experimental studies have also proven that lactoferrin stimulates the activity of lactoperoxidase. The susceptibility to *E. coli* and *Staphylococcus aureus* was shown, that microbial strains of *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Streptococcus uberis*, which have lactoferrin binding proteins on their surface, reduce the bactericidal effect of lactoferrin (Oudessa and Almeida, 2011; Günther et al., 2017). Also the data on immunomodulatory and neutralizing properties of lactoferrin were published. In particular, it was proved that the introduction of lactoferrin in the beginning of the inflammatory process in the mammary gland causes the extinction of the inflammatory reaction, in addition, it is able to bind to the lipopolysaccharides, while blocking their toxicogenic effect. The composition of Ferum-containing glycoproteins also includes transferrin. In

contrast to milk from mice and rabbits, the secret of the cows' mammary gland contains transferrin in low concentrations: from 1 mg/ml in colostrum and to 0.02–0.04 mg/ml in milk. Transferrin is delivered to the mammary gland from the blood, where its serum concentration is 4–5 mg/ml. In the acute mastitis (*E. coli*), the content of transferrin may reach 1 mg/ml, which is probably related to the Ferrum metabolism. Significant importance in the formation of antimicrobial protection plays lysozyme, the mechanism of action of which is based on the enzymatic hydrolysis of N-acetylmuramic bonds in the peptidoglycine complex (N-acetylmurmonic acid and N-acetylglucosamine) of the microorganism wall. The secretion of the mammary gland contains a small amount of lysozyme (0.13 µg/ml), which comes from the blood serum, and is excreted by macrophages (Zarrin et al., 2014; Law et al., 2017).

Serial clinical and experimental studies have traced the main stages of ontogenetic formation of cellular immunity of the mammary gland of cows. The total number of somatic cells in the secretion of the mammary gland of the primates varied from 180.78 ± 11.84 to 957.03 ± 12.03 thousand/ml ($P < 0.001$), which depended on the period of functioning of the mammary gland: in the colostrum, the secrecy prevailed ($56.00 \pm 1.90\%$) neutrophilic granulocytes, in the middle lactation period (3–5th month) the proportion of epithelial cells increased (from 29.51 ± 2.17 to $49.59 \pm 1.94\%$, $P < 0.001$), during the launch period, the population of polymorphonuclear neutrophil granulocytes practically recovered to the baseline level ($60.51 \pm 1.28\%$, $P < 0.001$) and then increased (to $66.07 \pm 1.61\%$, $P < 0.001$) during the dry period (Zhelavskij, 2014).

Fig. 1. Manifestation of cytochemical reactivity of neutrophil granulocytes (x 2000) secretion of the mammary gland of cows in reaction to ILL. Attraction (a) and phagocytosis (b) with microbe strain *Micrococcus lisodeikticus*

The cytochemical reactivity of intracellular lysozyme (ILL +) phagocytic cells in the secretion of the mammary gland (Fig. 1 A, B) of the primipar from the beginning of lactogenesis and in the middle period of lactation (3–5th month) was at a constant level (34.25 ± 0.81 and $37.33 \pm 0.83\%$, respectively).

However, at the end of lactation, during the onset and dry, with the development of involutional processes in the mammary gland of the primates, a sharp decrease (to $26.55 \pm 0.75\%$, $P < 0.01$) of the cytochemical reactivity of ILL + phagocytic cells was observed (Zhelavskij, 2015).

There is experimental evidence that lysozyme may exhibit antimicrobial activity as an independent microbial substance, and is associated with lactoferrin and opsonating antibodies (Prado et al., 2011).

Modern literature on the antimicrobial effects of lactoperoxidase, an enzyme protein of milk, which is excreted by neutrophilic granulocytes, should be taken into account. According to the latest data. Lactoperoxidase exhibits antimicrobial effect involving hydrogen peroxide (H_2O_2) and thiocyanate (SCN^-), catalyzing the formation of bactericidal mediators ($SCN)_2$, $HOSN^-$, HO_2SCN , HO_3SN , and endonuclease metabolites $^2SO_2^-$. Hypothiocyanide ($OSCN^-$) inhibits NADN-dependent glyceraldehyde-3-phosphate dehydrogenase, which is the cause of glycolysis in bacterial cells (Zarrin et al., 2014; Law et al., 2017; Herry et al., 2017; Rainard, 2017).

A similar trend was observed in the phagosomal activity of lysosomal cationic proteins (LCP). The growth of LCP activity in phagocytic cytoplasm during dry period was noted (62.62 ± 0.56 versus $53.81 \pm 0.78\%$, $P < 0.01$ in the colostrum period) and in the middle period (3–5th month) secretion milk against the background of a decrease in the percentage of the cytological index (CIL, $P < 0.001$) and the total cytochemical reactivity index in the second and third period of the functioning of the mammary gland (Zhelavskij, 2014; Zhelavskij et al., 2015).

The greatest reactivity of phagocytes was shown in the beginning of lacto-genesis of primates in the reaction to lysosomal cationic proteins (neutrophil activation index, IAN 0.92 ± 0.08), which acquired its maximum manifestation during the 3–5th month of lactation (1.36 ± 0.03 , $P < 0.001$), – during the period of the largest functional load of the mammary gland, and in the future (start, dry weight) gradually decreased ($P < 0.01$).

During colostrum, in the secretion of the breast of the firstborn, the amount of myeloperoxidase (MPO) -positive cells was $51.48 \pm 0.57\%$, IAN 0.87 ± 0.03 , and the CIL was $1.5 \pm 0.03\%$. During the 3–5th month of lactation, the total number of MPOs + phagocytic cells gradually decreased ($42.07 \pm 0.61\%$, $P < 0.001$) with a certain increase in IAN (1.03 ± 0.07 , $P < 0.001$) and cytological index ($1.82 \pm 0.07\%$, $P < 0.001$).

During the lactation, in the secret of the pruritic loss, there was also pronounced activation of the antimicrobial

potential of phagocytes in the NST-test. Thus, at the beginning of lactation, the number of formazanopositive neutrophil granulocytes in their colostrum was $32.96 \pm 0.93\%$, in the middle – gradually decreased to $12.8 \pm 1.01\%$ ($P < 0.01$), and at the end of lactation, the inverse wavelength increased: $16.67 \pm 0.55\%$ ($P < 0.01$) in start and $32.00 \pm 0.73\%$ ($P < 0.001$) in dry condition.

Our studies have proven that local breast immunity is ontogenetically formed, and the complete formation of cellular factors of immune defense in the womb takes place in several stages. Experimental studies showed a significant increase in the activity of LKB in the phagocyte cytoplasm in the dry period (62.62 ± 0.56 versus $53.81 \pm 0.78\%$, $P < 0.01$ in the colostrum period) and in the middle period (3–5 month) of secretion of milk against the background of a decrease in the percentage of cytological index (CIL, $P < 0.001$) and the total cytochemical reactivity index in the second and third period of the functioning of the mammary gland.

The greatest reactivity of phagocytes was shown in the beginning of lacto-genesis of primates in the reaction to lysosomal cationic proteins (neutrophil activation index, IAN 0.92 ± 0.08), which acquired its maximum manifestation during the 3-5th month of lactation (1.36 ± 0.03 , $P < 0.001$), – during the period of the largest functional load of the mammary gland, and in the future (start, dry weight) gradually decreased ($P < 0.01$).

Among the humoral factors in the protection of the mammary gland there is also xanthine oxidase – an enzyme that is part of the fat balls of milk. With its participation, the formation of nitrogen oxide is catalyzed by the emergence of strong reagents – free radicals, among which the superoxide anion is the most important one – the radical ($O_2^{\cdot-}$). In serial experiments it has been proved that at mastitis of bacterial etiology, with the participation of these reagents (*E. coli*, *Staphylococcus aureus*, *S. typhimurium*), the pH is sharply reduced, chemotaxis and phagocytosis are activated. Neutrophils, macrophages, natural killers (NK) and dendritic cells belong to cellular factors of protection of a mammary gland. It has been established that the total number and population of these cells in milk depends on the physiological state of the body. Most researchers indicate that at the beginning of lactation, the number of somatic cells reaches 1 million/ml, then gradually decreases during the first 7–10 days of lactation. Among somatic cells, the greatest percentage in these periods falls on neutrophilic granulocytes. The number of polymorphonuclear neutrophils in the secretion of the mammary gland increases during the period of secretion of colostrum and in the launch period (up to 40%). Especially the population of neutrophils increases with the development of the mastitis. 98% of neutrophilic granulocytes are mature (Prado et al., 2011; Zhelavskij, 2014; Rainard, 2017; Zhelavskiy, 2017).

Thus, in our serial studies, it was found that in the colostrum period the number of myeloperoxidase (MPO) – positive cells was $51.48 \pm 0.57\%$, IAN 0.87 ± 0.03 , and the CI of $1.5 \pm 0.03\%$. During the 3-5th month of lactation, the total number of MPOs + phagocytic cells gradually decreased ($42.07 \pm 0.61\%$, $P < 0.001$) with a

certain increase in IAN (1.03 ± 0.07 , $P < 0.001$) and of cytological index ($1.82 \pm 0.07\%$, $P < 0.001$).

Conclusions

In the process of ontogenetic development of the breast of the primates in parallel with the formation of secretory function of the mammary gland there is a gradual formation of cellular factors of its local defense. Oxygen-independent and Oxygen-dependent factors of phagocytic protection of the breast of the firstborn are not yet sufficiently formed, their activation begins with the colostrum period and undergoes a permanent oscillation throughout the lactation period.

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