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MILK TRYPSIN CLEAR INCREASES UNDER BOVINE MASTITIS SIMULTANEOUSLY WITH INFLAMMATION GENE EXPRESSION

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Abstract

Mastitis is one of the most serious problems in dairy farming. Mastitis often affects high-yielding cows, with a 10-15 % reduction in productivity and irreversible mammary gland dysfunction. In clinical course pathology has clear diagnostic signs. The main known methods of diagnostics of subclinical forms of mastitis (mastitis tests) are based on the determination of somatic cells in milk, the number of which correlates with inflammation, but the development of methods for early diagnosis of mastitis and pre-mastitis state of cows remains relevant. Biochemical parameters and morphological profiles of animal blood, expression of genes associated with inflammation are also examined in mastitis. However, the presence of enzymes in animal milk has not been fully studied. Trypsin is considered as a hormone-like substance capable of influencing metabolism and being a marker of inflammatory processes in animals and humans. Previously, we have shown the role of trypsin in experimental toxicosis of chickens and dietary changes. In the presented study we have for the first time revealed trypsin in the milk of cows, the increase in its activity in mastitis was established and compared with changes in other indicators used to assess the state of animals in pathology. The aim of the present work is to detect trypsin activity in milk of healthy and mastitis-affected cows and to determine the number of somatic cells in milk, relative expression of genes associated with inflammation, as well as morpho-biochemical blood parameters. The results obtained on Ayrshire cows (*Bos taurus*), 10 lactating cows without clinical signs of mastitis and 15 cows with clinical signs of mastitis (SGC Smena — a branch of the FSC VNITIP RAS, Moscow Province, 2022), showed that in the milk, the activity of genes associated with inflammation and the trypsin activity varied depending on the mammary gland health. In mastitis this index increased compared to the norm by 106.6 % ($p \le 0.05$), whereas trypsin activity in blood serum of healthy and mastitis cows had no significant differences. Of the biochemical parameters of cow blood, the most informative were the concentration of glucose, calcium and phosphorus. We found that in blood serum of mastitic cows the amount of glucose increases by 67.4% ($p < 0.05$), calcium by 38.8% ($p < 0.05$), the concentration of phosphorus, on the contrary, decreases by 23.8 % ($p < 0.05$) compared to healthy animals. In the blood morphological profile at mastitis leukocytosis is observed, there is a decrease in immunoreactivity by 42,5 % ($p < 0.05$), the ratio of lymphocytes and neutrophils by 20.4 % ($p < 0.05$), the number of eosinophils by 57.4 % ($p < 0.05$) and basophils by 33.3 % ($p < 0.05$), while the number of monocytes increases by 46.5 % compared to the control ($p < 0.05$). The expression of genes of monocyte chemotactic protein 1 and monocyte chemotactic protein 2 increased 5.5-fold, tumor necrosis factor alpha 3.9-fold, interleukin 4 and interleukin 8 2.9-fold and 14-fold, respectively, in cows with mastitis compared to healthy cows. Thus, we found that cow's milk contains trypsin, which is not inferior to the enzyme in the blood serum of animals in terms of activity (48.2±3.8 units/l). In inflammation of the mammary gland confirmed by instrumental and molecular genetic methods, trypsin activity in milk increases, which can be used in the development of diagnostic methods for pre-mastitis and early stages of mastitis.

Keywords: cows, mastitis, milk trypsin, mastitis diagnostic methods

Mastitis (inflammation of the mammary gland) is one of the most common diseases of dairy cattle. The pathology causes economic losses due to decreased milk production and poor milk quality [1-3]. To combat bovine mastitis and reduce the damage caused by this disease, a search is underway for more advanced and highly sensitive methods for diagnosing and treating the disease [4].

In ongoing studies, much attention is naturally paid to morphological and biochemical blood tests [5, 6], but the reliability of the parameters as prognostic indicators has not been proven [6]. Another modern approach is the assessment of the expression of genes associated with inflammation [7-9]. But even in this case, the results, regardless of connection with other indicators, are not yet considered as unambiguous [8].

It is known that active digestive enzymes are present in the blood of animals and humans [10-12]. The ability of enzymes to penetrate into the blood due to the structural features of pancreatic cells was described by Soviet scientists back in 1973 [13]. Blood enzymes are considered as possible markers in the diagnosis of mastitis in cows [14]. Such indicators include N-acetyl-beta-D-glucosaminidase, lactose, haptoglobin and serum amyloid A in cow milk [15]. It has previously been shown that only the alkaline phosphatase activity test is reliable in the early diagnosis of subclinical bovine mastitis, but not the lactate dehydrogenase and aspartate aminotransferase tests [15].

Some enzymes enter milk since they are synthesized in the cells of the mammary gland. Other enzymes are produced by various microorganisms found in milk, which, during vital activity, release substances that alter milk composition and properties. Lipase, lactase, phosphatase, reductase, peroxidase, catalase, trypsin, trypsinogen, and lysozyme were detected in human milk [14]. The scientific literature contains information on comparative analysis of enzyme activity in breast and cow's milk, as well as in human colostrum and regular milk [15]. There are evidences to support the view that lipase and ribonuclease probably enter milk from the blood; lysozyme is released from secretory epithelial cells; lactate and malate dehydrogenases, glucose-6-phosphate dehydrogenase and lactose synthetase are synthesized in the mammary gland; lipase, diastase, protease and lysozyme, stimulated by bile salts, are present in quantities sufficient to break down milk substrates [15]. Lipase activity in human milk is being studied [16].

Among the enzymes, in our opinion, trypsin, which is considered as a hormone-like substance, deserves detailed attention [17]. Trypsin affects metabolism and, we believe, can be a marker in the diagnosis of inflammatory processes in animals and humans [18]. Previously, we identified changes in trypsin activity during experimental chicken toxicosis [19] and depending on diets [20]. Although the transcriptomic responses of bovine mammary gland cells to mastitis-causing pathogens have been studied [8], the relationship between such signs of mastitis as the number of somatic cells in milk and the level of expression of immune genes associated with mammary gland inflammation is still unknown.

In this work, trypsin activity was discovered for the first time in cow's milk, and its changes during mastitis were established. New data have been obtained on the expression of inflammatory genes during cow mastitis. Thus, the transcriptional activity of the genes for monocyte chemotactic protein 1 increased by 5.5 times, monocyte chemotactic protein 2 by 9 times, tumor necrosis factor by 3.9 times, genes of interleukin 4 and interleukin 8 by 2.9 times and 14-fold.. Trypsin activity as a putative indicator of the mammary gland state is being discussed.

The goal of the work was to identify trypsin activity in the milk of healthy and mastitic cows and to investigate its probable association with the number of

somatic cells, the relative expression of inflammatory genes, and morpho-biochemical blood parameters.

Materials and methods. Physiological experiments were carried out on 25 Ayrshire cows (*Bos taurus*) at the farm of the Smena State Center, a branch of the Federal Scientific Center All-Russian Research and Technological Institute of Poultry — FSC VNITIP RAS, Moscow Province) in 2022. Group I (control) was healthy lactating cows without clinical signs of mastitis (n = 10), and group II with clinical signs of mastitis (n = 15). The mastitis was confirmed by kenotest, viscometric study (milk analyzer Somatos-Mini, LLC VPK Sibagropribor, Russia) and flow cytometry (automatic analyzer CombiFoss 7 DC, FOSS, Denmark). Flow cytometry determines the total somatic cell counts (SCC) and differential somatic cell counts (DSCC, the proportion of lymphocytes and polymorphonuclear neutrophils in the total number of cells) [21]. All tests were performed as recommended by the manufacturing companies. Milk was collected in the morning into sterile 15 ml tubes and no later than in 3 h, the enzyme activity was measured. Milk samples were prepared by centrifugation in microtubes for 5 min at 14,000 rpm (an Eppendorf 5430R centrifuge, Eppendorf, Germany). After centrifugation, the top layer containing fat was removed. For assay, the second layer after the fat was collected. Trypsin activity was measured (a biochemical analyzer SINNOWA 3000M (SINNOWA Medical Science & Technology Co., Ltd, China) by a kinetic method using Na-benzoyl-DL-arginine-p-nitroanilide (BAPNA, ACROS ORGANICS, Switzerland) as a substrate in accordance with description [22].

Blood was taken from the tail vein into vacuum tubes for collecting venous blood with a coagulation activator (filler silicon oxide SiO_2). To obtain comparable results, trypsin activity in blood serum was determined in the same way as in milk (a biochemical analyzer SINNOWA 3000M, SINNOWA Medical Science & Technology Co., Ltd, China) [22]. Blood biochemical parameters were examined (an automatic biochemical analyzer BioChem FC-120, High Technology, Inc., USA) with reagent kits for total protein, glucose, cholesterol, calcium, phosphorus, alkaline phosphatase assay (High Technology, Inc., USA).

Blood morphology was examined using an automatic hematology analyzer MicroCC-20Plus (High Technology, Inc., USA). Blood tests were performed on healthy cows and cows with mastitis (at least 2 times in each cow).

Gene expression analysis was performed using quantitative reverse transcription polymerase chain reaction (RT-PCR). Milk was sampled from each lobe of the udder of 6 cows (No. 1-3, clinically healthy animals, No. 4-6, animals with signs of mastitis), 24 samples in total. The samples were stabilized in IntactRNA solution (JSC Evrogen, Russia) according to the manufacturer's recommendations and stored at –20 °C.

The samples were homogenized (Precellys Evolution homogenizer, Bertin Technologies, France). Total RNA was isolated using the Aurum Total RNA kit (Bio-Rad, USA) according to the manufacturer's instructions. To obtain cDNA on an RNA template, a reverse transcription reaction was carried out with the iScript™ Reverse Transcription Supermix kit (Bio-Rad, USA). The gene amplification reaction (a DTlight detecting amplifier, NPO DNA-Technology, Russia) was carried out with primers described [23] using the SsoAdvanced™ Universal SYBR® Green Supermix kit (BioRad, USA) in accordance with the manufacturer's protocol [24]. The amplification mode and conditions for the analysis were as follows: 5 min at 95 °C (preliminary denaturation); 30 s at 95 °C, 30 s at 60 °C, 30 s at 70 °C (40 cycles) [25]. Relative expression was determined by the $2^{-\Delta\Delta CT}$ method [26]. The housekeeping gene *RPL19*, encoding ribosomal protein L19

(RPL19), was the reference gene.

Statistical processing included calculation of the mean value (*M*) and standard deviation (±SD) using Microsoft Excel. The significance of differences was assessed by Student's *t*-test. Differences were considered statistically significant at $p < 0.05$. Correlation analysis was performed according to Pearson using the Microsoft Excel computer program.

Results. Cows with clinical signs of mastitis, additionally confirmed by kenotest and viscometry, were selected for the study. Healthy animals without signs of mastitis served as controls.

SSC and DSSC values are used as biomarkers in breast health monitoring. These indicators were assessed in the milk of healthy and mastitic cows by flow cytometry.

1. The total number of somatic cells and the proportion of lymphocytes and polymorphonuclear neutrophils in the milk of healthy (group I) **and mastitic** (group II) **Ayrshire cows (*Bos taurus*)** (farm of the SGC Smena — a branch of the Federal Scientific Center VNITIP RAS, Moscow Province, 2022)

Parameter	Group I ($n = 10$)		Group II ($n = 15$)		t_d	p
	M±SD	*Cv*, %	*M*±SD	*Cv*, %		
Number of somatic cells, $\times 10^3$/ml	176.6±53.4	82.4	584.9±122.7	286.9	3.05	0.0089
Proportion of lymphocytes and polymorphonuclear neutrophils in the total number of cells, %	49.8±2.8	11.3	76.8±6.4	14.8	3.87	0.0046

N o t e. The total number of samples accounted for at least 20 in group I and at least 30.in group II.

In our experiment, in the milk of healthy cows, the number of somatic cells (SCC) was 176.6×10^3/ml, which is 3.31 times less ($p < 0.01$) than in the milk of animals with mastitis. The range of phenotypic variability (*Cv*, %) indicates significantly less variability of this indicator in the milk of healthy animals. The DSCC rate in healthy animals was 49.8%, while in the milk of sick animals it was 76.8%, or 1.54 times more. The difference between the compared groups was highly significant ($p < 0.01$). Noteworthy is the fact that in the compared groups the range of phenotypic variability for DSCC was close. Therefore, this indicator is more stable than the total number of somatic cells in milk.

2. Blood and milk trypsin activity in healthy (group I) **and mastitic** (group II) **Ayrshire cows (*Bos taurus*)** (*M*±SD; farm of the SGC Smena — a branch of the Federal Scientific Center VNITIP RAS, Moscow Province, 2022)

Parameter	Group I ($n = 10$)				Group II ($n = 15$)			
	Milk (by udder lobes)							
Activity, U/l	LA	LP	RP	RA	LA	LP	RP	RA
	51.0±10.3	51.0±7.7	47,0±6,2	44,0±6,5	111,0±18,3	76,0±11,5	82,0±12,1	130,0±14,5
On average	48.2±3.8				99.6±7.3*			
UH	1.1	1.1	1,2	1,3	0,5	0,7	0,6	0,4
On average	1.20				0.55			
	Blood serum							
Activity, U/l	57.9±2.5				52.4±3.1			

N o t e. UH — udder health coefficient, LA — left anterior lobe of the udder, LP — left posterior lobe of the udder, RP — right posterior lobe of the udder, RA — right anterior lobe of the udder. Samples from each animal were
* Differences from group I are statistically significant at $p < 0.05$.

We detected trypsin activity both in blood serum and in cow's milk (Table 1). Within the sample, the activity of this enzyme turned out to be a stable indicator. To assess the health of the mammary gland, we propose the following formula: ZMZH = TK/TM, where ZMZH is the coefficient of breast health; TK — trypsin activity in the blood, units/l; TM — trypsin activity in milk, units/l. The breast index of 1.1 and above indicates normal state of the mammary gland; when the breast index is < 1.1, a deviation from the norm occurs, caused by inflammation in the mammary gland.

The results showed that there were no significant differences in trypsin activity in the blood serum of healthy and mastitic cows. However, trypsin activity in milk clearly corresponded to the health status of the mammary gland, fluctuations from the average sample value were due to milk fat. Thus, in cows with mastitis, trypsin activity in milk increased by 106.6% ($p < 0.05$) compared to values in healthy animals.

Analysis of the correlation between trypsin activity in milk and in blood serum in cows showed that in healthy animals this relationship is stable, moderate and positive ($r = 0.43$; $p < 0.05$), and in case of mammary gland pathology it became moderate negative ($r = -0.45$; $p < 0.05$). Therefore, in animal husbandry practice, to diagnose the early stage of mastitis, an udder health assessment coefficient (UH) can be proposed, consisting of two interrelated indicators. The coefficient is calculated as the ratio of trypsin activity in blood to trypsin activity in fresh animal milk according to the formula given above.

To assess the general health of the animals, we performed biochemical blood tests (Table 3).

3. Blood biochemical parameters in healthy (group I) **and mastitic** (group II) **Ayrshire cows (*Bos taurus*)** (M±SD; farm of the SGC Smena — a branch of the Federal Scientific Center VNITIP RAS, Moscow Province, 2022)

Parameter	Group I (n = 10)	Group II (n = 15)
Total protein, g/l	102±5.2	92±1.9
Albumin, g/l	52±4.2	62±3.2
Glucose, mmol/l	2.2±0.14	3.7±0.12*
Cholesterol, mmol/l	3.6±0.65	4.0±0.28
Calcium, mmol/l	2.2±0.06	2.5±0.07*
Phosphorus, mmol/l	2.1±0.18	1.6±0.13*
Alkaline phosphatase, U/l	249±12.7	180±30.9

N o t e. The total number of samples accounted for at least 20 in group I and at least 30.in group II.
* Differences from group I are statistically significant at $p < 0.05$.

In this case, the most informative indicators were the blood levels of glucose, calcium and phosphorus. The results showed that in the blood of cows with mastitis, the amount of glucose increases by 67.4% ($p < 0.05$), calcium by 38.8% ($p < 0.05$), while the phosphorus content, on the contrary, decreases by 23.8% ($p < 0.05$) compared to healthy animals.

To assess the immune status of animals, we examined blood morphological blood parameters (Table 4).

4. Hematological parameters in healthy (group I) **and mastitic** (group II) **Ayrshire cows (*Bos taurus*)** (M±SD; farm of the SGC Smena — a branch of the Federal Scientific Center VNITIP RAS, Moscow Province, 2022)

Parameter	Group I (n = 10)	Group II (n = 15)
White blood cells (WBC), $\times 10^9$/l	5.0±0.42	9.6±0.93*
Neutrophils (Neu), %	43.4±3.51	49.5±6.78
Lymphocytes (Lym), %	44.9±1.30	40.7±4.60
Monocytes (Mon), %	4.3±0.40	6.3±0.83*
Eosinophils (Eos), %	6.8±0.53	2.9±0.18*
Basophils (Bas), %	0.6±0.06	0.4±0.04*
Red blood cells (RBC), $\times 10^{12}$/l	5.5±0.09	5.4±0.12
Hemoglobin concentration (HGB), g/l	91.0±0.51	85.0±1.22*
Hematocrit (HCT), %	26.6±0.25	25.0±0.38*

N o t e. The total number of samples accounted for at least 20 in group I and at least 30.in group II.
* Differences from group I are statistically significant at $p < 0.05$.

The number of leukocytes in the blood of cows with mastitis increases by 92.0% ($p < 0.05$). To determine the level of stress, we used as a basis the Harkavi index [27], calculated as the ratio of the relative content of lymphocytes to the relative content of neutrophils. Despite the fact that the percentage of neutrophils and lymphocytes are within physiological norms, the calculated index indicates

stress in animals. In healthy cows, the indicator is 1.03, in cows with signs of mastitis 0.82. The decrease in the coefficient is associated with a decrease in the number of lymphocytes by 9.4%. Calculation of the immunoreactivity index (IIR) according to D.O. Ivanov (2014) by the formula IIR = (L + E)/M, where L are lymphocytes, E are eosinophils, M are monocytes [28] showed a 42.5% decrease in this indicator under mastitis. With inflammation of the mammary gland, we noted a decrease in the number of eosinophils (by 57.4%; $p < 0.05$), monocytes (by 31.5%; $p < 0.05$) and basophils (by 33.3%; $p < 0.05$).

Oxidation processes in cows with mastitis were less intense due to a 6.6% ($p < 0.05$) decrease in the level of hemoglobin that carries oxygen to cells. In animals with mastitis, the hematocrit decreased by 6.0% ($p < 0.05$) compared to healthy cows, which also negatively affects metabolism.

5. Relative gene expression in milk of healthy (group I) **and mastitic** (group II) **Ayrshire cows (*Bos taurus*)** (*M*±SD; farm of the SGC Smena — a branch of the Federal Scientific Center VNITIP RAS, Moscow Province, 2022)

Group	*MCP-1*	*MCP-2*	*TNF-α*	*INF-γ*	*IL2*	*IL4*	*IL8*	*Casp6*
I ($n = 3$)	1	1	1	1	–	1	1	–
II ($n = 3$)	5.48±0.68	9.17±0.67	3.85±0.51	0.72±0.33	–	2.85±0.26	14.17±1.60	–

N o t e. A milk sample was taken from each lobe of the udder, a total of 24 samples in the experiment. Dashes mean that gene expression was not detected.

When comparing the expression of the genes *MCP-1*, *MCP-2*, *TNF-α*, *INF-γ*, *IL2*, *IL4*, *IL8*, *Casp6* in healthy and mastitic cows, we found an increase in the transcriptional activity of almost all genes associated with the development of inflammation. It is known that inflammation can result from the interaction of multiple regulatory pathways or biological processes [29]. However, at present, knowledge about the expression of inflammatory and regulatory cytokines in cells present in cow's milk is insufficient. Previous studies have shown that proinflammatory cytokines are often considered as promising biomarkers of mastitis, including specific ones for determining the status and etiology of the disease [8]. In particular, it has been reported that during the early stages of mastitis, the level of inflammatory cytokines increases faster than the total number of somatic cells in milk [9]. However, the use of the number of somatic cells in milk as a selection trait to increase resistance to mastitis in cattle has given limited results [30], therefore, information on molecular markers of susceptibility/resistance to mastitis is considered promising for identifying cattle genetically resistant to mastitis [31-33].

The etiology of mastitis, in addition to mechanical damage during milking, is often associated with various infections that affect the host body in the early stages of the disease, causing, in particular, the secretion of cytokines. Moreover, the production of different cytokines in response to different infections is not the same, which can serve as a differentiating factor in the etiology of mastitis [34-36]. Thus, the development of mastitis is most often associated with bacterial infections, but viruses, microscopic algae and fungi can also act as pathogens [37-40]. In particular, algae of the genus *Prototheca* have been reported as the third most common causative agent of mastitis after members of the genera *Streptococcus* and *Staphylococcus* [41].

Internal mammary ductal epithelial cells play a key role in recognizing mastitis-causing pathogens through toll-like receptors (TLR2 and TLR4) [42, 43]. TLRs influence the transcription factor NF-κB which controls the expression of immune response, apoptosis, and cell cycle genes, particularly tumor necrosis factor-α (TNF-α) genes and the interleukins IL1β, IL6, and IL8 [44-46]. It is well known that serum cytokines such as interferon, tumor necrosis factor α, IL17, IL6, and IL4 play a key role in inflammatory processes, suggesting their possible

involvement in the pathological process of mastitis in cattle [47, 48].

Cytokines are important for intercellular communication. Known processes that are stimulated or inhibited by cytokines include cell differentiation, proliferation, remodeling, degeneration, regeneration, and even cell death. It has been reported that in mastitis, along with an increase in the number of somatic cells, the level of secreted cytokines (interleukins IL1, IL2, IL4, IL5, IL6, IL8, IL10 and IL12) in milk increases [49].

In our study (see Table 5), in cows with mastitis, there was an increase in the expression of monocyte chemotactic protein 1 and monocyte chemotactic protein 2. These are cytokines belonging to the group of CC-chemokines. In sick cows, the expression of *MCP-1* increased 5.5-fold, *MCP-2* 9-fold. Monocytes play a leading role in inflammation. The accumulation of monocytes is due to their adhesion and migration under the influence of chemoattractants, in particular the recently described chemotactic cytokines (chemokines) [50, 51]. The monocyte-specific chemokine MCP-1 (monocyte chemoattractant protein 1) is synthesized by activated monocytes/macrophages and vascular wall cells and, by binding to its receptor CCR2, regulates the adhesion and migration of monocytes on matrix proteins and endothelium. MCP-2 has unique functional properties compared to other chemokines, including MCP-1. Other research teams have shown in in vitro models of mastitis that bovine mammary epithelial cells can express the chemokines CXCL6 (also called GCP2) and CCL8 (also called MCP2) in response to certain bacterial cell components [52, 53]. In general, the expression of monocyte chemotactic protein 1 and monocyte chemotactic protein 2 in mastitis has been little studied, so our data are of significant interest in connection with the issues under discussion.

The expression of tumor necrosis factor α in cows with mastitis increased by 3.9 times compared to healthy ones (see Table 5). TNF-α is a proinflammatory cytokine produced primarily by macrophages. Depending on the location of its release and the receptor with which it binds, TNF-α can perform various functions, e.g., to stimulate synthesis of other cytokines and cause inflammatory reactions, to control vital processes in the cell and maintain tissue homeostasis [54, 55]. In this regard, in combination with other cytokines, TNF-α plays an important clinical role in cattle, mediating immune inflammatory reactions (mastitis, endotoxic shock, endometritis). Cytokines, particularly TNF-α, participate in the development of metabolic diseases, e.g., acidosis [56]. TNF-α is involved in inflammation [57] and regulates a number of physiological functions, including appetite, fever, energy metabolism, and endocrine activity [58]. Various agents such as viruses, parasites, bacteria, and endotoxins induce TNF-α production [59].

We also found that the expression level of interleukins 4 and 8 in cows with mastitis increased by 2.9 times and 14 times, respectively (see Table 5). Interleukins are polypeptides produced by cells involved in immune and inflammatory responses [60].

The main producers of IL4 in the mammary glands of cattle are T- and B-lymphocytes, eosinophils and basophils, mast cells, plasma cells [60, 61], as well as epithelial cells, which together form the basis of the type II immune response [62]. IL4 has been reported to regulate innate immunity and have an inhibitory effect on IFN-β in dairy cows [60]. In our study, along with increased IL4 expression, we noted a trend towards increased expression of IFN-β, a cytokine secreted by various cells involved in innate and adaptive immune responses [63], as well as by antigen-presenting cells involved in the elimination of pathogens [64, 65]. Interestingly, in other studies, the IL4 content in milk during mastitis, on the contrary, decreased (66), therefore, this issue requires further study.

Interleukin 8 (IL8) [67] is an inflammatory cytokine produced by a variety of cell types, e.g., lymphocytes, neutrophils, monocytes, macrophages, and epithelial cells [68], including bovine mammary epithelial cells [69]. At the site of inflammation, IL8 is involved in the recruitment and activation of neutrophils [70]. During the acute phase of coliform mastitis, the concentration of IL8 in cow's milk increases significantly [71]. The chemotactic activity of IL8 was detected in the mammary gland secretions of cows with mastitis during intramammary infection with *Staphylococcus aureus*, but not in healthy cows [72]. Thereof, interleukin 8 is believed to provide the infiltration of neutrophils into mammary gland secretions during mastitis. Interleukin 8 also alters milk protein composition by inhibiting milk-specific protein secretion and the influx of whey proteins [72]. Overall, IL8 is considered a potent mediator of inflammation and is also involved in the recruitment of leukocytes to sites of infection [73]. Polymorphisms of the *IL8* gene under mastitis are being studied [74, 75]. It was reported that one of the polymorphisms, the +472 A>G in *IL8* is associated with a high SSC in the milk of cows infected with *S. aureus* [76].

Since blood trypsin activity is related to the content of nitric oxide metabolites [77], it is possible that trypsin is involved in the inflammatory response in breast tissue. Moreover, there is a method for diagnosing mastitis by the content of nitrite (NO^{2-}) and nitrosothiols (RSNO) in milk [4]. The method for detecting these compounds in biological objects is widely described in special literature [78]. Evidences suggest that trypsin is not only a pancreatic enzyme, but also a signaling molecule involved in metabolism and maintaining the health of organs and tissues.

For a long time it was believed that trypsin is synthesized only in the pancreas. Results from a study of human and mouse nonpancreatic tissue samples showed [79] that the trypsin gene is expressed at high levels in the pancreas, spleen, and significantly in the small intestine. In situ hybridization and immunohistochemical analysis revealed trypsin expression in epithelial cells of skin, esophagus, stomach, small intestine, lung, kidney, liver, extrahepatic bile ducts, in spleen cells and neurons. In the spleen, trypsin is found in macrophages, monocytes and lymphocytes in the white pulp, in the brain — in nerve cells of the hippocampus and cortex [79]. Such a wide distribution in the body suggests a general role of trypsin in maintaining normal functions of epithelial cells, the immune defense system, and the central nervous system [79].

Receptors (PAR2) activated by trypsin have also been discovered [80]. Trypsin is the main PAR2 activating protease that initiates inflammatory signaling [81]. The PAR2 receptor is located on apical and basolateral membranes of intestinal epithelial cells [82, 83] and can be stimulated by trypsin, tryptase, and bacterial proteases [84]. PAR2 is also present in the membrane of immune cells, stromal and endothelial cells. Systemically, PAR2 stimulation promotes blood coagulation, adhesion, and leukocyte extravasation [85]. Thus, it can be assumed that the increase in trypsin activity in the milk and blood of cows with mastitis is associated with activation of PAR2 receptors in mammary epithelial cells and blood leukocytes.

In this work, we for the first time identified trypsin activity in the milk of healthy cows and noted its significant increase in mastitis, which indicates a connection between trypsin and the inflammation in the mammary gland. Previously, we studied the role of trypsin in experimental toxicosis of chickens [19] and under changes in diets [20]. Scholar publications note the multiplicity of trypsin localization [80] and functions [84, 85], including signaling [17]. Therefore, trypsin activity may serve as a marker of homeostasis disorders. The connection between trypsin activity in milk, on the one hand, and mastitis, including its physiological and

biochemical signs, on the other hand, has not been previously studied.

We additionally performed biochemical and morphological blood analyzes and measured SCC, DSCC and the expression level of the main immune genes in milk. It turned out that in cows with mastitis, along with a 2-fold increase in trypsin activity, SCC, DSCC and the expression of the *MCP-1*, *MCP-2*, *TNF-α*, *IL4* and *IL8* genes increased manifold. The revealed multiple increases in trypsin activity and gene expression give reason to believe that by combining these indicators, it is possible to develop a test with sensitivity sufficient for early diagnosis of subclinical forms of mastitis and pre-mastitis state. Comparison of these indicators over time, starting with pre-mastitis period, and with mastitis of different etiologies will provide new knowledge about the mechanisms of development and course of this pathology. At the next stage of research, we also plan to determine the expression of the trypsin gene in healthy and mastitic cows.

Thus, the results of our research draw us the following conclusions. In healthy cows, trypsin is discovered in milk for the first time. In the milk, its activity amounted to 48.2±3.8 units/l, being comparable to that in blood serum. During inflammation of the mammary gland, somatic cell count (SCC) in milk increases 3.3-fold ($p < 0.01$) vs. healthy animals, and trypsin activity increases 2.0-fold. In the blood of mastitic cows, the glucose content is 67.4% higher ($p < 0.05$), total calcium 38.8% higher ($p < 0.05$), while the phosphorus, on the contrary, 23.8 % lower ($p < 0.05$) than in healthy animals. Therefore, for these blood biochemical parameters, the body's response to the pathology is significantly lower than for SCC and trypsin in milk. In mastitic cows, the ratio of lymphocytes and neutrophils decreases by 20.4%, immunoreactivity by 42.5%, the number of eosinophils by 57.4%, basophils by 33.3%; the number of monocytes increases by 46.5%. This is also much lower than changes in SCC and trypsin activity in milk under mastitis. Compared to healthy animals, in cows with mastitis, the expression of genes associated with inflammation was much higher, in particular 5.5-fold for monocyte chemotactic protein 1, 9-fold for monocyte chemotactic protein 2, 3.9-fold for tumor necrosis factor, 2.9-fold for interleukin 4 and 14-fold for interleukin 8. The large differences identified in trypsin activity in milk and the expression of genes associated with inflammation in norm and in mastitis create prospects for the development of a diagnostic test with sensitivity sufficient for the early detection of subclinical forms of mastitis and pre-mastitis state.

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