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PROSPECTS FOR THE APPLICATION OF EXTRACELLULAR PROTEINASES OF MICROMYCETE *Aspergillus ochraceus* IN THE TREATMENT OF MASTITIS IN COWS

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Abstract

Cow mastitis, the etiological agents of which are pathogenic and opportunistic microorganisms, is considered one of the diseases that cause significant economic damage to dairy farming with a risk to the health of dairy consumers. With bacterial infections of the mammary gland in cows, the formation of protein exudates occurs. Proteinases that reduce the severity of the inflammatory response are included in the treatment regimens for various pathologies in medicine, but this practice is limited in veterinary medicine. In this work, we proved that a drug based on *Aspergillus ochraceus* proteinase increases the effectiveness of antibiotic therapy for cow mastitis. The aim of the study was to experimentally evaluate the possibility of using *A. ochraceus* BKM F-4104D micromycete proteinase in veterinary medicine. The studies were performed on lactating black-and-white cows (*Bos taurus*) with milk productivity for the previous lactation of 6900-7110 kg, which were divided into two groups (n = 16 each). All experimental animals were intracisternally injected with an anti-mastitis drug based on the beta-lactam antibiotic amoxicillin, clavulonic acid and prednisolone (ACP) at a dose of 3.0 g (one syringe dispenser) once per day for 3-4 days until the disappearance of clinical signs of mastitis. In the second group, 12 hours before the use of the ACP preparation, animals were additionally intracisternally administered a drug with the working name PAO-1. PAO-1 is an oil suspension containing 4.0 g (syringe-dosing device) extracellular proteinase of the micromycete *A. ochraceus* BKM F-4104D as an active ingredient. This proteinase is able to degrade heterogeneous protein substrates in a wide range of environmental conditions, which can increase the effectiveness of the etiotropic therapy of bovine mastitis. The condition of the breast, morpho-biochemical status were evaluated before treatment, after treatment-and 7-10 days after the end of the administration of the drugs. It was found that the combined use of an antimicrobial agent and a preparation based on the proteinase of the micromycete *A. ochraceus* BKM F-4104D was accompanied by the recovery of 93.8 % of cows with clinical mastitis, which is 12.5 % higher ($p < 0.05$) than when using only the antimastitis drug ACP. Recovery of animals was characterized by normalization of morpho-biochemical status. The amount of β-globulins increased by 14.2 % ($p < 0.05$), triglycerides by 31.4 % ($p < 0.05$), creatinine decreased by 24.2 % ($p < 0.05$) compared to animals treated with ACP therapy. Endogenous intoxication and lipid peroxidation decreased, e.g., the concentration of malonic dialdehyde decreased by 40.4 % ($p < 0.00005$), medium-weight molecules by 46.3 % ($p < 0.00005$), NOx 3.6-fold ($p < 0.00005$), endogenous intoxication index was 33.7 % lower ($p < 0.005$) compared to sick animals. The activity of the enzymatic and non-enzymatic components of the antioxidant defense increased, the concentration of vitamin A increased by 36.8 % ($p < 0.005$), vitamin E by 32.8 % ($p < 0.05$), vitamin C by 39.2 % ($p < 0.005$), catalase activity increased by 39.4 % ($p < 0.005$), glutathione peroxidase activity increased by 30.6 % ($p < 0.005$) compared to sick animals. Optimization of protein, lipid and mineral metabolism occurred. After the end of the therapeutic course, the number of somatic cells and their

composition in the secret of the udder was normalized and there was no pathogenic microflora, which confirmes the complete clinical recovery of the cows. Our findings indicate that *Aspergillus ochraceus* BKM F-4104D micromycete proteinase which has high anticoagulant and fibrinolytic activity, can be very promising for the creation of domestically produced enzymatic veterinary drugs competitive in the world market.

Keywords: proteinases, *Aspergillus ochraceus* BKM F-4104D, PAO-1 drug, mastitis, cattle, enzyme preparations, combination therapy

In many countries, cow mastitis is considered a common pathology that leads to significant economic losses in dairy farming [1-5]. Among the main causes of mastitis are the effects of pathogenic and opportunistic microorganisms [6-9].

Most drugs for the treatment of mastitis contain antibiotics as an active ingredient. However, their use has led to the emergence of drug-resistant strains of microorganisms [5], the persistence of biofilm resistance of *Staphylococcus aureus* to antibiotics [1], and the isolation of multidrug-resistant microbial isolates from cows with clinical mastitis [9, 10]. In addition, antibiotics can have a toxic effect on the fetus during pregnancy, cause allergic reactions, dysbacteriosis and immunodeficiency in young animals [11]. Therefore, reducing the frequency and/or frequency of antibiotic use is a task that requires more and more attention. It can be solved by increasing the effectiveness of antibiotic therapy [12, 13].

The consequences of bacterial infections of the reproductive organs and mammary gland in cows include the formation of blood clots and protein exudates, which plays a significant role in the development of infertility and a decrease in milk production [14, 15]. To solve the problem in therapeutic practice, drugs containing proteinases are successfully used, which reduce the severity of the inflammatory response, which is manifested in the normalization of microcirculation and the reduction of edema, and the improvement of tissue trophism [16]. This is due to the proven presence of fibrinolytic, immunomodulatory and other effects in proteinases [16, 17].

The fibrillar proteins fibrin and collagen are difficult to hydrolyze substrates that require proteolytic enzymes with specific activity for their cleavage [18]. In clinical practice, preparations based on such proteinases are used to eliminate blood clots, purulent masses, and necrotic tissues in the affected area [18]. According to a number of studies, among such enzymes, proteinases of micromycetes, especially representatives of the genus Aspergillus, which produce several types of enzymes with a directed action of limited proteolysis, stand out with high activity and efficiency [18-20]. In this regard, the proteases of filamentous fungi, which can effectively cleave fibrin, collagen, elastin, keratin, and other fibrillar proteins, can be of great practical importance for veterinary medicine.

It is also known that many processes of homeostasis are regulated by various forms of proteases, the activity of which, in turn, is in a complex interdependence with the action of the oxidant and antioxidant systems [17].

Proteolytic enzymes, having a range of biological effects, can affect the condition of animals during antibiotic therapy of mastitis, in particular, their hematological status and sanitary characteristics of the udder secretion. However, such studies are not well represented in the scientific literature.

In our work, we compared the results of a morpho-biochemical blood test, determination of markers of the activity of the lipid peroxidation-antioxidant protection system, microbiological and morphobiochemical analysis of mammary gland secretion in two treatment regimens and proved that a drug based on *Aspergillus ochraceus* proteinase, which is able to cleave heterogeneous protein substrates in a wide range of environmental conditions, increases the effectiveness of antibiotic therapy for mastitis in cows.

The purpose of our study is an experimental and clinical evaluation of the

possibility of using Aspergillus ochraceus BKM F-4104D micromycete proteinase to increase the effectiveness of treatment of cow mastitis in combination with etiotropic agents.

Materials and methods. All procedures performed in the study were previously reviewed and approved at a meeting of the bioethical commission of the ARVRIPP&T RAS and corresponded to type A (manipulations with animals that do not cause pain or cause minimal pain and discomfort). The personnel participating in the experiment were trained in the correct and humane treatment of animals in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes (ETS 123, Strasbourg, 1986); Directive 2010/63/EU of the European Parliament and of the Council of the European Union of 22 September 2010 on the protection of animals used for scientific purposes; Guide for the Care and Use of Laboratory Animals, Washington (DC) (1996); Code of Ethics of a Veterinarian of the Russian Federation, recommended at the XIII Moscow International Veterinary Congress of the Association of Practitioners of Veterinary Doctors of Russia (2005).

In the experiment, we used a drug with the provisional name PAO-1 (developed at the ARVRIPP&T), which contains recombinant *Aspergillus ochraceus* BKM F-4104D micromycete proteinase with anticoagulant and fibrinolytic properties. Proteinase was expressed in *Escherichia coli* BL 21 strain (DE3) in soluble form and inclusion bodies and subsequently in high yield was obtained using refolding and affinity chromatography on Ni-NTA-agarose [21].

Lactating cows (*Bos taurus*) of black-motley breed of tie-down housing (n = 32; Agrotech-Garant LLC Rostoshinsky, Ertilsky District, Voronezh Province, February-March 2021) with milk production for the previous lactation of 6900-7110 kg divided into two groups of 16 cows each to determine the therapeutic efficacy of drugs. All cows (groups I and II) were intracisternally injected with an anti-mastitis drug based on the β-lactam antibiotic amoxicillin, clavulonic acid and prednisolone (ACP) at a dose of 3.0 g (one syringe dispenser) 1 time per day for 3-4 days until disappearance of clinical signs of mastitis. Animals from group II, 12 hours before the use of the ACP preparation, were additionally injected once intracisternally with the drug PAO-1 (oil suspension, 4.0 g, one dosing syringe).

The clinical state of the mammary gland was assessed before treatment, during treatment (3-4 days) and 7-10 days after the administration of the last therapeutic dose of ACP. In addition, blood was taken from 5 cows from each group from the jugular vein into Green Vac-Tube vacuum tubes (Green Cross, South Korea) for laboratory studies of morpho-biochemical parameters (the amount of total protein and its fractions, urea, creatinine, glucose, total lipids, triglycerides, cholesterol, activity of alkaline phosphatase ALP, aspartate aminotransferase AsAT, alanine aminotransferase AlAT, γ-glutamyl transferase GGT, total calcium, inorganic phosphorus, copper, zinc, manganese, magnesium, selenium, protein-bound iodine PBI) and activity markers of the lipid peroxidation, i.e., the antioxidant defense system (LPO-AOD) (concentrations of malonic dialdehyde MDA, activity of glutathione peroxidase GPO and catalase Cat, content of vitamins A, E, C, medium-weight molecules MWM, stable metabolites of nitric oxide NOx) [22, 23]. Morphological studies were performed on an ABX Micros 60 hematological analyzer (HORIBA ABX SAS, France). Biochemical studies were performed on a Hitachi-902 analyzer (Hitachi, Japan).

The secret of the udder from cows with catarrhal mastitis was collected according to the instructions [24, 25]. Bacteriological studies of the udder secretion (in 5 animals in the group), the study of the cultural, morphological and biochemical properties of the isolated microorganisms were carried out in accordance with the recommendations [25]; the number of somatic cells was determined

according to GOST 23453-2014 using a somatic cell analyzer in milk DCC (DeLaval, Sweden) in accordance with the instructions for the device [26], the composition of the leukocyte population was determined by microscopy of udder secretion preparations stained according to Romanovsky-Giemsa (microscope Bioscope-1, LOMO, Russia). The content of circulating immune complexes (CIC) in udder secretion was determined by PEG precipitation using a UV 1800 spectrophotometer (Shimadzu, Japan) [27].

Statistical data processing was performed using the MedCalc 15.8 program (MedCalc Software, Ltd., Belgium). The mean values (*M*) and standard errors of the means (±SEM) were determined. Statistical significance was assessed using the nonparametric Mann-Whitney U-test, differences were considered statistically significant at $p < 0.05$.

Results. In group I of cows with mastitis, when using only the antimicrobial drug ACP, 81.3% of animals recovered (when taken into account by udder shares, this figure was 83.3%). In group II, with the combined use of antimicrobial and enzyme preparations, the therapeutic efficacy was 93.8 and 94.7%, respectively. Therefore, the additional use of the enzyme preparation significantly ($p < 0.05$) increases the therapeutic effect by 12.5%.

The positive effect of the complex use of antimicrobial and enzyme preparations was confirmed by our results of a study of the morphological and biochemical characteristics of the blood of animals. The recovery of cows treated with the antimicrobial agent ACP (Table 1) was accompanied by a decrease in the content of -globulins in the blood by 31.4% ($p < 0.005$), creatinine by 9.9%, ALP activity by 37.7% ($p \leq 0.00001$), AsAT by 17.6% ($p < 0.05$), AlAT by 47.2% ($p \leq 0.00002$), GGT by 8.9% with an increase in albumin content by 24.1% ($p < 0.05$), triglycerides by 1.5 times ($p \leq 0.00001$), the amount of total calcium by 48.4% ($p < 0.005$) compared to the state before the start of treatment. The combined use of ACP and PAO-1 in the treatment of clinical mastitis led to a more pronounced increase in the albumin fraction of the protein (by 25.5%, $p < 0.05$), β-globulins by 19.8% ($p < 0.05$), the amount of triglycerides by 2 times ($p < 0.00005$), cholesterol by 15.1%, total calcium by 54.3% ($p < 0.005$), copper by 12.8% ($p < 0.05$), zinc by 13.2% ($p < 0.05$) with a decrease in the α-globulin fraction of the protein by 38.0% ($p < 0.005$), creatinine by 31.7% ($p < 0.005$), ALP activity by 38.7% ($p \leq 0.00005$), AsAT by 27.3% ($p < 0.005$), AlAT by 53.1% ($p < 0.00005$), GGT by 19.8% ($p < 0.05$). In the group of animals that received the antimicrobial drug in combination with PAO-1, an increase in the amount of β-globulins by 14.2% ($p < 0.05$), triglycerides by 31.4% ($p < 0.05$) occurred with a decrease in the content of creatinine by 24.2% ($p < 0.05$) relative to the indicators in the group of animals that were administered only an antibacterial drug.

An analysis of the prooxidant-antioxidant status of cows showed that mastitis occurs against the background of intensified lipid peroxidation, which indicates the presence of oxidative stress, endogenous intoxication also increases and AOD decreases. This was indicated by high concentrations of MDA, MWM, NOx and the value of the endogenous intoxication index (EII) at low values for the enzymatic and nonenzymatic AOD units (Table 2).

After a course of antibiotic therapy with the use of ACP, the recovery of animals was accompanied by a decrease in the blood index of endogenous intoxication by 16.0%, the content of medium-weight molecules by 23.7% ($p < 0.00005$), malondialdehyde by 25.4% ($p < 0.05$), NOx by 57.7% ($p < 0.00005$) with an increase in the content of vitamin E by 14.6%, vitamin C by 11.0%, vitamin A by 23.7% ($p < 0.05$), GPO activity by 14.9%, Cat by 18.2%.

1. Morphobiochemical blood parameters in black-motley cows (*Bos taurus)* with mastitis before and after treatment with antibiotic and enzyme preparation (*M*±SEM, Agrotech-Garant LLC Rostoshinsky, Ertilsky District, Voronezh Province, February-March 2021)

Parameter	Reference values	Before treatment (a basal level)	After treatment	
			group I (n = 5)	group II (n = 5)
Erythrocytes, $\times 10^{12}$/l	4.8-7.0	5.72±0.37	5.81±0.41	5.79±0.45
Hemoglobin, g/l	90-140	116.3±6.1	115.6±7.2	120.4±7.9
Total protein, g/l	72-86	78.4±4.00	80.3±4.80	82.4±4.11
Albumins, %	38-50	37.7±2.30	46.8±3.12*	47.3±2.84*
α-Globulins, %	12-20	24.5±1.92	16.8±0.81**	15.2±1.30**
β-Globulins, %	10-16	10.1±0.41	10.6±0.31	12.1±0.38**▲
γ-Globulins, %	25-40	27.7±1.11	25.8±0.90	25.4±1.13
Urea, mmol/l	3.0-6.7	3.82±0.21	3.94±0.19	3.99±0.22
Creatinine, μmol/l	40-180	119.4±7.7	107.6±5.0	81.6±4.60**▲
Glucose, mmol/l	2.1-3.8	3.12±0.12	3.45±0.22	3.51±0.13
Total lipids, g/l	1.4-5.6	4.24±0.21	4.31±0.19	4.61±0.18
Triglycerides, mmol/l	0.25-0.70	0.34±0.01	0.51±0.01***	0.67±0.02***▲
Cholesterol, mmol/l	1.3-5.5	3.45±0.22	3.56±0.19	3.97±0.22
ALP, E/l	100-200	289.6±5.5	180.3±4.1***	177.5±3.9***
AsAT, U/l	10-50	85.4±4.11	70.4±3.90*	62.1±3.6**
AlAT, U/l	10-30	38.6±1.64	20.4±1.53***	18.1±1.42***
GGT, U/l	7-15	19.2±1.1	17.5±1.34	15.4±1.09*
Calcium total, mmol/l	2.25-3.15	1.88±0.12	2.79±0.15**	2.90±0.19**
Phosphorus inorganic, mmol/l	1.45-2.3	1.72±0.11	1.80±0.17	1.74±0.11
Copper, μmol/l	12.6-30.0	14.8±0.58	15.1±0.71	16.7±0.44*
Zinc, μmol/l	46.2-77.0	44.7±3.1	48.6±3.9	50.6±1.15*
Manganese, μmol/l	2.7-4.6	2.82±0.21	2.99±0.17	3.19±0.16
Magnesium, mmol/l	0.8-1.25	0.87±0.09	0.91±0.08	0.95±0.07
PBI, μg%	4-8	5.36±0.31	5.41±0.37	5.65±0.29

N o t e. For a description of the groups, see the Material and methods section. AP, AsAT, AlAT, GGT stand for alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, γ-glutamyl transferase, respectively, PBI — protein-bound iodine.

*, **, *** Differences from the basal level are statistically significant at $p < 0.05$, $p < 0.005$, $p \le 0.00005$, respectively.

▲ Differences from group I are statistically significant at $p < 0.05$.

2. Parameters of the lipid peroxidation-antioxidant protection system and endogenous intoxication in black-motley cows (*Bos taurus*) with mastitis before and after treatment with an antibiotic and an enzyme preparation (*M*±SEM, Agrotech-Garant LLC Rostoshinsky, Ertilsky District, Voronezh Province, February-March 2021)

Parameter	Reference values	Before treatment (a basal level)	After treatment	
			group I (n = 5)	group II (n = 5)
MDA, μmol/l	0.8-1.2	3.32±0.24	2.32±0.18*	1.98±0.07***
MSM, U OD_{254}	< 0.3	0.41±0.01	0.29±0.01***	0.22±0.01***
EII, CU		16.9±1.24	13.7±0.91	11.2±0.63**
GPO, μmol G-SH/(l · min · 10^3)	20.0-35.0	14.3±1.24	16.2±1.41	18.8±1.23*
Cat, мμmol H_2O_2/(l · min · 10^3)	30.0-40.0	41.1±3.37	48.1±3.84	57.7±4.21**▲
Vitamin A, μmol/l	0.84-2.78	1.33±0.12	1.67±0.11*	1.82±0.09**
Vitamin E, μmol/l	15.0-30.0	12.3±1.12	14.1±1.33	16.2±1.23*
Vitamin C, μmol/l	34.1-85.2	20.0±1.92	22.3±1.80	27.7±2.31*
Sw, μmol/l	1.0-1.5	1.02±0.05	1.24±0.07	1.35±0.06**
NOx, μmol/l	40-120	128.6±6.66	52.8±2.56***	36.3±2.23***▲

N o t e. For a description of the groups, see the Material and methods section. MDA, MWM, EII, GPO, Cat stand for malondialdehyde, medium-weight molecules, endogenous intoxication index, glutathione peroxidase, and catalase, respectively.

*, **, *** Differences from the basal level are statistically significant at $p < 0.05$, $p < 0.005$, $p \le 0.00005$, respectively.

▲ Differences from group I are statistically significant at $p < 0.05$.

In the blood of animals that additionally received PAO-1, there were more pronounced changes vs. those before treatment (a basal level). Thus, the blood content of NOx decreased by 3.6 times ($p < 0.00005$), malondialdehyde by 40.4% ($p < 0.00005$), medium-weight molecules by 46.3% ($p < 0$, 0005), the index of endogenous intoxication decreased by 33.7% ($p < 0.005$) with an increase in the content of vitamin C by 39.2% ($p < 0.05$), vitamin A by 36.8% ($p < 0.005$),

vitamin E by 32.8% ($p < 0.05$), selenium by 32.4% ($p < 0.005$), glutathione peroxidase activity by 30.6% ($p < 0.05$), catalaseby 39.4 % ($p < 0.005$), which indicates a decrease in the rate of lipid peroxidation and activation of enzymatic and non-enzymatic components of antioxidant protection (see Table 2). In group II, compared to group I, catalase activity increased by 20.2% ($p < 0.05$), and the NOx decreased by 31.3% ($p < 0.05$).

The complex use of ACP and PAO-1 preparations favorably affected the cytological composition of milk cells (Table 3).

3. Immune and cytomorphological parameters of the udder discharge in black-motley cows (*Bos taurus*) before and after treatment with an antibiotic and an enzyme preparation (M±SEM, Agrotech-Garant LLC Rostoshinsky, Ertilsky District, Voronezh Province, February-March 2021)

Parameter	Reference values	Before treatment (a basal level)	After treatment	
			group I ($n = 5$)	group II ($n = 5$)
Somatic cells, $\times 10^3$/ml	< 200	4620.2±718.1	351.4±56.8***	189.7±22.1***▲
Lymphocytes, %	20-30	5.1±0.35	25.1±4.9**	25.8±3.2**
Neutrophils, %	12-20	91.1±7.71	41.2±3.7**	19.1±2.7***▲
Macrophages, %	55-65	3.8±0.25	33.7±3.1***	55.1±5.2***▲
Lysozyme, μg/ml	0.5-1.8	2.01±0.03	0.71±0.07***	0.55±0.04***
Circulating immune complexes, g/l	0.05-1.0	0.253±0.04	0.099±0.01**	0.061±0.01**▲

N o t e. For a description of the groups, see the Material and methods section.
*, **, *** Differences from the basal level are statistically significant at $p < 0.05$, $p < 0.005$, $p \leq 0.00005$, respectively.
▲ Differences from group I are statistically significant at $p < 0.05$.

The content of somatic cells in the secretion of the mammary gland in animals of group I (ACP) at the end of the experiment decreased by 13.0, in group II (ACP + PAO-1) by 24.7 times, and this decrease was almost 1.9 times higher compared to group I.

Cytomorphological analysis of somatic cells of the secret from the affected lobes of the mammary gland of cows with mastitis found that neutrophils were predominant, the content of which was 89.7 and 92.5% in animals of groups I and II, respectively. At the end of treatment in the milk of cows from group II, the number of lymphocytes was close to normal (25.8%) while the number of neutrophils (19.1%) and macrophages (55.1%) approached the optimal value. In the milk of cows treated only with ACP, an increased content of neutrophils (41.2%) and a decreased content of macrophages (33.7%) were noted, the content of lymphocytes was close to optimal (25.1%). After treatment, the observed changes in parameters relative to the background in both groups, as well as in group II vs. group I (except for the number of lymphocytes and the lysozyme level) were statistically significant (see Table 3). A 1.62-fold excess of the concentration of CIC in animals of group I compared to cows that received additional PAO-1 may indicate a violation of the permeability of the vascular wall, an increase in the inflammatory response, the release of lysosomal enzymes and suppression of T lymphocytes [27].

Bacteriological studies of the secret of the mammary gland of cows showed that at the end of treatment with ACP, the microflora was not isolated in 60.0% of cases, *Staph. aureus* was detected in 20.0% of cases and E. coli in 20.0%. In the milk of cows from group II, subjected to complex treatment (ACP + PAO-1), no microflora was found.

It is known that some proteolytic enzymes realize a therapeutic effect through the influence on the inflammatory process, vascular-platelet hemostasis and immune responses [17]. Proteolytic enzymes improve tissue trophism by destroying protein formations and fibrin clots in the area of inflammation, as well as reducing platelet aggregation, thereby preventing the transition of a chronic inflammatory process to a recurrent stage [28, 29]. Enzymes can act as natural highly

active inflammation modulators to speed up the healing process [28].

A number of studies have shown the ability of extracellular proteinases of micromycetes of the genus Aspergillus to exhibit hydrolytic properties and activate protein C and factor X in blood plasma [30]. Proteinase, an activator of blood plasma protein C isolated from the culture liquid of *A. ochraceus* VKM F4104D, which is a serine proteinase [31] with high biological activity and anti-inflammatory action, was obtained [32].

Our clinical trials have shown the effectiveness of an experimental preparation based on *A. ochraceus* micromycete proteinase in the complex treatment of mastitis in cows. Additional use of PAO-1 increased the therapeutic effect by 12.5% which was confirmed by the data of morpho-biochemical studies. Changes in homeostasis indicators in the process of recovery of cows with the combination of the antimicrobial drug ACP with PAO-1 indicate, on the one hand, a decrease in the inflammatory response and functional load on the liver and kidneys due to a decrease in endogenous intoxication, on the other hand, the normalization of protein, lipid and mineral metabolism.

Lipid peroxidation is an important metabolic factor in both normal and pathological conditions [33]. The pathogenesis of many animal diseases is based on the intensification of lipid peroxidation processes, which leads to disruption of cellular energy exchange, protein synthesis, inhibition of membrane-dependent enzymes due to the accumulation of a number of toxic products (MDA, conjugated dienes, ketodienes) [34, 35]. Mastitis proceeded with oxidative stress and an increase in endogenous intoxication, as indicated by the increase in the concentration of MDA and MWM, high EII, low values characterizing the activity of the enzymatic (Cat, GPO), non-enzymatic (vitamins A, E and C) AOD and high NOx content. When analyzing the prooxidant-antioxidant status of recovered animals, it was found that in cows that additionally received PAO-1, positive changes were more pronounced. So, in these animals, the concentration of MDA, MWM decreased, the EII decreased with an increase in the content of vitamins A, E, C and the activity of Cat and GPO, which indicates a decrease in the intensity of lipid peroxidation and activation of the enzymatic and non-enzymatic AOD.

The recovery of animals after a course of ACP in combination with PAO-1 was accompanied by a more significant decrease in the blood NOx content (the difference was 31.3%), which also indicates a weakening of oxidative stress, since NO is able to act both as a powerful pro-oxidant and participate in endogenous antioxidant protection [36, 37].

The detected changes may be due to the fact that the enzymatic activity of the proteinase improves microcirculation in inflammatory foci, reduces vascular porosity, provides more complete removal of damaged tissues and pus clots from the milk ducts and, consequently, faster elimination of pathogenic microorganisms and bacterial toxins with milk, while antibiotics reached the inflammatory focus more quickly [28, 38].

The clinical recovery of cows after the end of the therapeutic course is confirmed by the normalization of the number and composition of somatic cells, as well as the absence of pathogenic microflora in the udder secretion with the additional use of PAO-1 in combination with etiotropic treatment.

So, the results of experimental and clinical studies allow us to draw the following conclusions. The use of the micromycete *Aspergillus ochraceus* BKM F-4104D proteinase in combination with etiotropic treatment for mastitis of cows provides a significant increase in the effectiveness of antibiotic therapy (by 12.5%, $p < 0.05$) and the complete release of the mammary gland from mastitis pathogens *Staphylococcus aureus* and *Escherichia coli*. Changes in the blood

morphobiochemical parameters during treatment indicates the normalization of metabolic processes, a decrease in the toxic effect of endogenous metabolites (the content of malondialdehyde decreased by 40.4% at $p < 0.00005$, medium-weight molecules by 46.3% at $p < 0, 00005$) and stimulation of the enzymatic and non-enzymatic components of the antioxidant system (an increase in the content of vitamin A by 36.8% at $p < 0.005$, vitamin E by 32.8% $p < 0.05$, vitamin C by 39.2% at $p < 0.005$, catalase activity by 39.4% at $p < 0.005$, glutathione peroxidase activity by 30.6% at $p < 0.005$). This reduces the metabolic load on the liver and kidneys of animals. Combined therapy induces a more pronounced modulation of pro- and antioxidant status compared to etiotropic treatment, i.e., a 3.6-fold decrease in NOx content ($p < 0.00005$). This is probably due to a decrease in inflammation at the site of infection, since in the secret of the udder there was a significant decrease in the number of somatic cells (neutrophils to the greatest extent, up to 19.1% at $p < 0.05$) and the content of circulating immune complexes (up to 0.061 g/l at $p < 0.05$). The absence of pathogenic bacteria in the udder secretion with the proteinase preparation means that the decrease in the inflammatory response is also associated with a decrease in the action of endotoxins produced by bacteria and pathogen-associated molecular patterns. We believe that the reduction of endo- and exogenous intoxication, as well as the activation of reparative processes in the focus of infection due to fibrinolytic remodeling of the extracellular matrix under the action of proteinase, can contribute to the normalization of morphobiochemical parameters. Thus, the proteolytic functions of the studied enzyme preparation are likely to underlie the increase in the effectiveness of therapy. The *A. ochraceus* BKM F-4104D micromycete proteinase with anticoagulant and fibrinolytic properties may be very promising in the development of drugs for veterinary medicine, since its use enhances the antibiotic effect and, possibly, will reduce the amount of the drug itself.

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