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Pathomorphological changes in the organs of the peripheral immune system in mycobacteriosis of cattle

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Abstract. Conducting routine allergy tests of cattle with a simultaneous sample in TB-prone farms is necessary to confirm the stage of the epizootic process. The purpose of the research – to explore the pathomorphological changes in cows upon detection of para allergic reactions during diagnostic testing for tuberculosis. The diagnosis of tuberculosis in animals was performed comprehensively using all methods for tuberculosis control provided for in the guidelines – epidemiological, clinical, allergic, pathological, histomorphological, bacteriological and biological. It allowed establishing that the reactions to tuberculin in cows were of a paraallergic nature, caused by nontuberculous mycobacteria or so-called atypical mycobacteria. It was determined that cows that reacted positively to tuberculin and an atypical mycobacterial allergen and were sent for slaughter for diagnostic purposes did not demonstrate pathological changes characteristic of tuberculosis. However, pathological changes (granulomatous foci, atrophy of lymphoid and epithelioid tissue, hyperplasia of varying intensity and nature, dotted haemorrhages in the cortical zone) were observed in many lymph nodes, including mediastinal, mesenteric, pharyngeal and mandibular lymph nodes, indicating an active infectious process in the body of animals caused by nontuberculous mycobacteria. Therewith, several changes were observed in the lymph nodes during histomorphological examination: development of primary nodules of lymphocytes and reticular tissue in reactive centres, degradation and necrosis of lymphocytes, the establishment of cellular detritus in the centre of the nodules and capsules of epithelioid elements around the nodules, etc., which can be used to confirm the patterns of pathology development and, indirectly, the mechanism of sensitisation of the cow's body, and therefore the nature of paraallergic reactions in animals. The obtained results are important for the timely detection and identification at the histomorphological level of a complex of changes in the organs and tissues of cows due to the penetration of nontuberculous mycobacteria, which is the key to the successful diagnosis of primary foci of mycobacteriosis and their effective prevention

Keywords: tuberculosis; tuberculin; antigen of atypical mycobacteria; infectious process; atypical mycobacteria; allergic reactions; diagnostics

Introduction

Mycobacteriosis is an infectious disease of animals and humans caused by pathogens belonging to a large group of nontuberculous mycobacteria (NTMB) or so-called atypical mycobacteria (AMB).

Currently, it has been identified more than 180 species of mycobacteria, of which 30-60 are etiological agents of mycobacteriosis (Adjemian *et al.,* 2017, 2018; Armstrong *et al.*, 2023).

The relevance of mycobacteriosis was raised after an increase in mortality of AIDS patients with generalised NTMB infection was noted (Ose *et al.*, 2021; Thomas *et al.,* 2023). The increase in the incidence of mycobacteriosis is associated with immunosuppression resulting from glucocorticoid and cytostatic therapy, treatment with tumour necrosis factor α blockers (Taira *et al.*, 2018), a decrease in the spread of tuberculosis (Madzinga *et al.*, 2022), and improved methods of isolation and identification of NTMB (Haworth *et al.*, 2017; Kok *et al.,* 2022).

Recently, scientists in many countries around the world have been focusing on the growing problem of NTMB infection in mammals. Thus, since 2000, North America, Europe, New Zealand, and Australia have recorded a

steady increase in the prevalence of NTMB (Prevots & Marras, 2015). The authors state that the prevalence of NTMB in the setting of pulmonary disease continues to increase, and the number of such cases is lower in Europe than in North America and Australia. Global surveys of tertiary care facilities in East Asia (Japan, South Korea, Taiwan) indicate a high and growing prevalence of NTBIs. In several African countries, NTMBs were detected in 4.2-15% of cases of suspected tuberculosis and in 18-20% of individuals with "chronic" suspected multidrug-resistant tuberculosis.

The first documented cases of NTMB pulmonary infections in the English medical literature from Ecuador were reported by Echeverria *et al.* (2023). In Denmark (Dahl *et al.*, 2023), the incidence rate fluctuated from 1.3 (2013) to 2.5 (2021) per 100 thousand people**.** Scientists conclude that there is a highly positive linear correlation between the incidence of NTMB over the years (r=0.75, p=0.010), which indicates an upward trend. Vonasek *et al.* (2023) reported a stable annual incidence of NTMB infection in Wisconsin (USA) in 2011-2018. The authors report more than 90% of cases of respiratory tract NTMB infection, the vast majority of which were caused by *M. avium complex*. Among the fast-growing mycobacteria, skin and soft tissue pathogens predominated and were considered secondary respiratory pathogens.

Within the genus *Mycobacterium*, of the more than 170 different species of mycobacteria, the largest number (more than 160 species) belonged to nontuberculous mycobacteria. They occurred in the environment, were highly resistant and adaptable to growth and development. They frequently infected humans and animals (Cvetnić *et al.*, 2018).

Nontuberculous mycobacteria, in particular: *M. scrofulaceum, M. fortuitum, M. chelonei, M. kansasii, M. marinum, M. xenopi, M. vaccae, M. phlei, M. diernhoferi, M. smegmatis, M. peregrinum* were isolated from biomaterial from animals (cattle, pigs) and poultry (pigeons, sparrows, chickens), and from environmental objects (soil, feed, water, manure, etc.) (Sumbul & Doymaz, 2020; Pavlik *et al.,* 2021; Ulmann *et al.,* 2021).

Lesions of the gastrointestinal tract and respiratory organs in cattle caused by *Mycobacterium caprae* were detected by Ozturk-Gurgen *et al.* (2020). Scientists believe that the changes they observed reflect a continuous primary period of infection, without the lesions typical of the secondary (post-primary) period, which is typical for humans and bovine TB.

Circulation of a significant number of NTMBs (according to the International Classification of Bacteria, ICD-10, 1993; synonyms: ACM – atypical acid-fast mycobacteria; NTM – nontuberculous mycobacteria; ACB – acid-fast bacteria; MAS – *M. avium complex*) in the environment and specifically in cattle herds results in parallergic reactions to tuberculin in animals, which can be up to 50% in herds (Zavgorodnii *et al.*, 2016; Zavgorodnii *et al.*, 2021).

Bovine mycobacteriosis and tuberculosis are diseases that are widespread in the world (Busol *et al.*, 2014; Zimpel *et al.*, 2020; Varela-Castro *et al.*, 2022). Some scientists believe that one of the factors of nonspecific reactions to tuberculin may be latent microbiism caused by human and avian mycobacteria or atypical mycobacterial species (Oleksyuk & Levkivskyi, 2009).

Despite the fact that Ukrainian livestock production is free from TB (Dyachenko *et al.*, 2005) tuberculin-reactive animals continue to be found in many TB-prone farms (Zavgorodnii *et al.*, 2016). Thus, for each specific case of detection of tuberculin-reactive animals, a comprehensive approach to timely and effective diagnosis should be professionally applied (Zavgorodnii *et al.*, 2016).

Animal tuberculosis causes significant economic losses to the livestock industry and pos-

es a threat to human health, and therefore the fight against this anthropophagous disease is of epidemiological importance (Golovko *et al.,* 2016). Epizootic control of bovine tuberculosis is conducted comprehensively. However, preference is given to timely lifetime diagnosis using the allergic method (Kassych *et al.*, 2004; Dyachenko *et al.*, 2005).

Disclosure of the mechanism of paraallergic reactions to tuberculin requires a comprehensive approach to the exploration of the infectious process in mycobacteriosis, in particular, immunological, biochemical, pathophysiological and pathohistological aspects of the interaction between the pathogen and the macroorganism. The establishment of pathological and histomorphological changes in the organs and tissues of NTBM-infected animals will allow disclosing of the mechanism of sensitisation and the nature of these reactions to tuberculin more fully. The relevance of such researches is confirmed by the fact that in recent years there have been a rather small number of reports in the scientific literature on the influence of NTMB at the organ, tissue and cellular levels in cattle.

The purpose of the research was to determine pathological and pathohistological changes in the body of cattle with mycobacteriosis.

Materials and Methods

In one of the cattle farms in the Volyn region, more than ten cows were found to be positive for tuberculin during a routine tuberculosis test. Based on the analysis of the epizootic situation in this farm in 2017-2022, it was found that the farm's cattle herd was free of tuberculosis pathogen, cows' reactions to tuberculin were parallergic in nature, and the dairy herd was infected with NTMB of several species, which cause latent mycobacteriosis (Boyko *et al.,* 2020a; Boyko *et al.,* 2020b). Some features of the infectious and epizootic processes of this disease have been identified (Boyko *et al.*, 2021). A comprehensive research of infectious and epizootic processes in mycobacteriosis of dairy cows has been launched in June 2017. During this time, 14 allergy studies were performed, 13 diagnostic slaughters of reactive animals were conducted, and biomaterial from 57 cows was subjected to comprehensive (pathological, histological, microbiological and biological) examination (Table 1). However, despite a significant number of studies conducted in this area, the nature of paraallergic reactions to tuberculin has not been definitively established.

Table 1. Register of diagnostic slaughter of cows that tested positive for tuberculin or allergen atypical mycobacteria, conducted during 2017-2022

No. mon	Date of slaughter	Place of slaughter	Number of animals	Inventory number of animals	Pathological changes typical for tuberculosis
1	24.07.2017	Horodok	10	8121, 6634, 1010, 6443, 1019, 5952, 6605, 1018, 4543, 3497	Not detected
2	18.08.2017	Ivanychi	2	3464, 1412	Not detected
3	10.10.2017	Horodok	3	3415, 9838 and 794504	Not detected
4	05.06.2018	V. Mosty	3	3427, 7899, 6571	Not detected
5	12.12.2018	Horodok	3	794572, 3458, 7378	Not detected
6	26.06.2019	Dubno	3	5588, 124539, 124558	Not detected
7	25.10.2019	Horodok	5	7364, 9905, 5810, 1729, 8112	Not detected
8	23.06.2020	Horodok	4	3495, 4615, 6571, 5510	Not detected
9	25.10.2020	Horodok	6	3433, 5991, 5895, 5856, 4594, 5620	Not detected

Table 1. Continued

No. mon	Date of slaughter	Place of slaughter	Number of animals	Inventory number of animals	Pathological changes typical for tuberculosis
10	26.04.2021	Horodok	4	5894, 4283, 5647, 794591	Not detected
11	03.11.2021	Horodok	4	7837, 9772, 089896, 7894	Not detected
12	27.04.2022	Horodok	4	9438, 794588, 9819, 944265	Not detected
13	02.11.2022	Horodok	6	107890, 5587, 9373, 429894, 9408, 1721	Not detected
Total			**57**		

Source: *author's development*

The epizootic situation was controlled by double allergy testing of the entire animal population with a simultaneous test using purified tuberculin for mammals in standard solution (hereinafter referred to as tuberculin) and allergen dry purified from atypical mycobacteria (NTM) with a solvent of mycobacterial allergens (Kassych *et al.*, 2004).

Cows with the most pronounced reactions to both tuberculin and NTM were selected for diagnostic slaughter. Animals were slaughtered on day 5-7 after the allergy research.

The pathological examination was conducted in a modernly equipped slaughterhouse. To identify changes typical for tuberculosis, all lymph nodes were examined: mandibular, pharyngeal, cervical, forearm, scapular, knee fold, supraorbital, mediastinal, mesenteric and portal, as well as parenchymal organs: liver, kidneys, spleen, heart, lungs and both lining of the lung pleura, udder parenchyma, mesentery, small and large intestine.

For histological examination, the pharyngeal, mandibular, mediastinal, portal, mesenteric, supraorbital, pre-orbital, scapular and knee fold lymph nodes, and altered areas of the large intestine were selected.

The sampled material was fixed in a 10% solution of neutral formalin. After thorough washing, the material was placed in a homogenised paraffin mixture. The blocks were cut serially, with a cut thickness of 5 µm. Sections were stained with hematoxylin and eosin. Photographs of histological specimens were taken using a ZEISS Primo Star 3 microscope (Germany), a colour digital camera Axiocam 208 (Germany) and Zen ZEISS software at the Department of Histology and Medical Biology of Lesya Ukrainka Volyn National University. The research was conducted according to the ARRIVE guidelines for reporting experiments using live animals.

Results and Discussion

In previous studies, it was established that the first manifestations of parallergic reactions in heifers and in first-calf cows of the dairy farm of the examined farm were caused by sensitisation of the animals' organism to NTMB. The latter caused sensitisation of the animals' organisms and an infectious disease – mycobacteriosis (Boyko *et al.,* 2020a; Boyko *et al.,* 2020b).

The study of the infectious process became possible provided that after each scheduled allergy test of cows, a clinical examination of reactive animals was performed, a commission selection of cows for diagnostic slaughter, and post-slaughter examination of organs and tissues, sampling of biomaterial and its complete microbiological and histomorphological examination. Using such comprehensive research allowed for a more objective assessment of certain aspects of the infectious process in cows caused by NTMB.

New methods have been developed for the detection and identification of NTMBs, including molecular genetic methods (polymerase chain reaction – PCR), which are faster and easier to use than methods based on the isolation of pure cultures (Shevchenko *et al.,* 2019).

However, due to the widespread distribution of most NTMB species in the environment, the definitive diagnosis of an infectious disease caused by these mycobacteria cannot be based on culture or PCR alone. Thus, pathological and pathohistological findings are required to establish the final diagnostic result for mycobacteriosis (Prevots & Marras, 2015).

Analysing the data presented in Table 1, it can be noted that out of 57 cows with positive reactions to allergens, no pathological changes characteristic of tuberculosis were found in any of the examined animals. However, in all cows slaughtered for diagnostic purposes, several other pathological changes were noted, in particular: granulomatous foci, atrophy of lymphoid and proliferation of epithelioid tissue, hyperplasia of varying intensity and nature, and dotted haemorrhages in the cortical zone of the lymph nodes (Fig. 1-2).

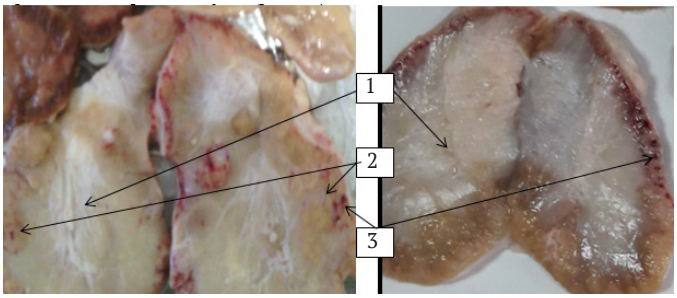

Figure 1. Pathological changes in the supraclavicular lymph nodes

Notes: *1 – degeneration with complete atrophy of the parenchyma of the cerebral layer; 2 – parenchymal remnants in the cortical layer; 3 – haemorrhages in the cortical zone*

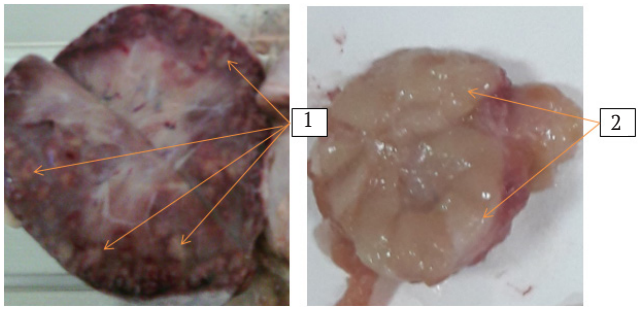

Figure 2. Pathological changes in mediastinal (1) and mandibular (2) lymph nodes

Notes: *1 – granulomatous foci in the mediastinal lymph node; 2 – parenchymal hyperplasia in the submandibular lymph node (pronounced parenchymal greasiness)*

Notably, the pathological changes presented in these figures were detected only in the sagittal section of the lymph nodes examined. Therewith, there was no crunching characteristic of tuberculosis, which is caused by the calcification of tuberculous nodules, which confirms the absence of tuberculous changes. Thus, the pathological picture presented in the figures, on the one hand, confirms the presence of an infectious process in the body of the reacting cows, which is mainly localised in the lymph nodes, and on the other hand, indicates

the absence of a tuberculosis process caused by *Mycobacterium bovis* or *M. tuberculosis* in the body of the reacting animals.

In some lymph nodes, changes in the colour of the parenchyma (reddening or dark grey discolouration), sebaceousness, and replacement of the node parenchyma with connective tissue were detected, indicating the presence of atrophic processes, as evidenced by the growth of connective tissue, which displaces lymphoid tissue, as a result of which the lymph node gradually loses its physiological function (Fig. 1). Hyperplastic processes in the lymph nodes were manifested by the growth of lymphoid tissue, and infiltration with lymphocytes, which is evidence of the presence of latent inflammatory processes in the surrounding tissues of the nodes themselves, provided that foreign agents – bacteria, fungi, etc. In the described case, these are acid-fast NTMB species that sensitised the immune system to their antigens, which manifested as allergic reactions to NTM, and to antigens of other mycobacterial species, including tuberculosis with parallergic reactions to tuberculin.

Analysing the manifestation of the infectious process in the lymph nodes depending on their location, pathological changes were most frequently detected in mediastinal, mesenteric (especially those that collect lymph from the large intestine), supraclavicular, pharyngeal, mandibular and much less frequently in the lymph nodes of the knee fold, pre-lobar and scapular lymph nodes. The established facts suggest that the detected regional intensity of the infectious process indicates a high intensity of a load of tissues and organs of the respiratory and digestive systems with NTMB antigens. It, in turn, indicates the priority of aerogenic (hyperplasia of mediastinal lymph nodes – (Fig. 2.1) and nutritional (hyperplasia of mesenteric, pharyngeal and mandibular lymph nodes – (Fig. 2.2) ways of entering these microorganisms into the body of animals.

The severity of pathological changes decreases over time. Thus, in cows slaughtered at the first diagnostic slaughter (2017-2019), pathological changes in the lymph nodes are more pronounced than those found in cows slaughtered for diagnostic purposes in subsequent years, when the number of responding animals began to decrease markedly (Fig. 3).

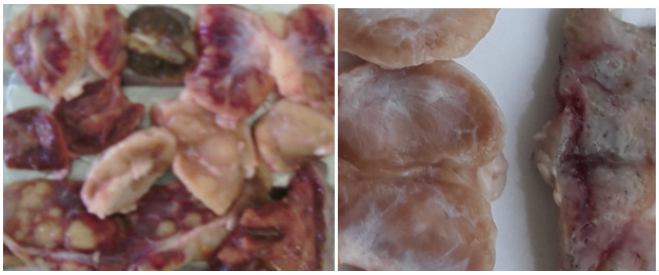

Figure 3. Pathological changes in lymph nodes in cows at the first (2017) diagnostic slaughter (A) and the last (2022) (B)

Notes: *A – pronounced granulomatosis and hyperplasia of mediastinal, mandibular and pharyngeal lymph nodes at the first diagnostic slaughter of reactive cows. B – slight replacement of the parenchyma of the suprarenal node with loose connective tissue*

It may indicate that at the beginning of the research, the epizootic process of mycobacteriosis in cattle in the herd was characterised by a long and uncontrolled development. As a result, pathological reactions prevailed over physiological ones, which caused greater damage to

the structure of the organs where they occurred and, ultimately, led to more pronounced pathological changes in the lymph nodes.

At the first diagnostic slaughter, in addition to lymph nodes, pathological changes in the large intestine were found in most slaughtered cows. These changes were mainly localised in the initial part of the colon and resembled paratuberculous enteritis. In particular, the affected areas of the intestine (30 to 60 cm long) had a mild thickening on the serous membrane. In the section, the entire affected area of the intestine was characterised by a pronounced folding of the mucous membrane, which resembled the surface of the cerebral hemispheres (Fig. 4). The detected pathological changes in the large intestine indicate local penetration of NTMB into the submucosal layer of the colon. The latter is rich in lymphoid elements in the form of solitary follicles and peyer's plaques, where the infectious process between NTMB and the animal's body occurs. This fact gives reason to believe that the nutritional route of mycobacteria entering the body is one of the main ones. Propagating in the intestinal submucosa, NTMBs provide both the entobiotic phase of their population and are both an endogenous and exogenous source of mycobacterial infection.

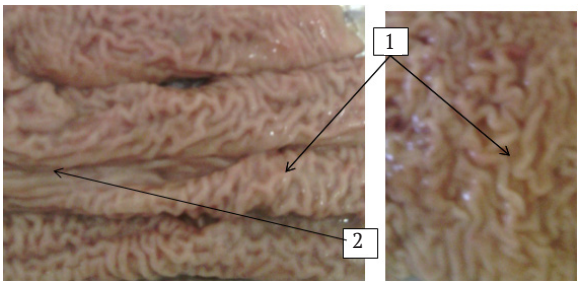

Figure 4. Pathological changes in the proximal colon

Notes: *1 – transverse pathological folding of the colon caused by an infectious process; 2 – longitudinal physiological folding of the colon.*

In addition to the transverse folding of the large intestine as a local manifestation of the infectious process in mycobacteriosis caused by NTMB, most animals demonstrated local pathological changes in the colon in the form of diverticula. These diverticula can be easily identified from the serous membrane as a bulging wall thickening in this area with a darker colour of the serous membrane. The increased folding of the mucous membrane and its thickening are clearly visible in the section (Fig. 5).

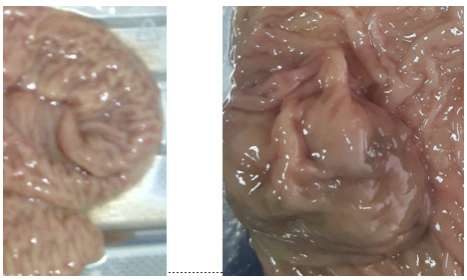

Figure 5. Pathological changes in the large intestine

Notes: *A - diverticulum in the wall of the colon mucosa; B - bulging of the colon mucosa into the intestinal lumen*

The detected pathological changes are not typical for tuberculosis, but they indicate the presence of local foci of the infectious process of varying intensity in the body of cows that reacted positively to tuberculin and NTM.

The isolation of acid-resistant NTMBs from lymph nodes and affected areas of the large intestine suggests that allergic reactions to tuberculin and NTM are the result of sensitisation of the body to these microorganisms (Zavgorodnii *et al.,* 2016). To better understand the infectious process caused by NTMB, histomorphological changes in the lymph nodes taken from each animal killed for diagnostic purposes were thoroughly explored. The most characteristic histological changes found in lymph nodes from cows that were positive for PPD-tuberculin and NTM are presented below (Figs. 6-10).

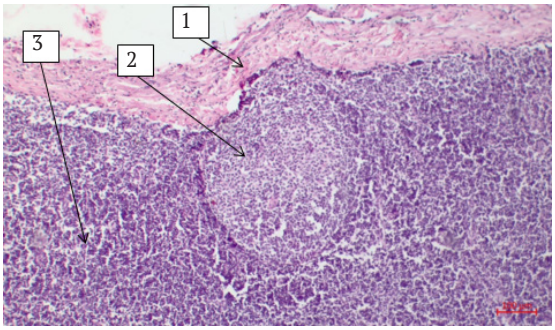

Figure 6. Structure of the lymph node (hematoxylin and eosin staining)

Notes: *1 – lymph node capsule; 2 – lymph follicle; 3 – cortical zone*

Fig. 6 presents the microscopic structure of a lymph node in the physiological standard. As can be observed from Figures 7 and 8, the place of influence of NTMB pathogenicity factors on the immune system of infected animals is the lymphatic follicles, where the development of primary nodules of lymphocytes and reticular tissue was observed at the beginning of the infectious process. Subsequently, degradation was observed in them (cytoplasm swelling, increased eosinophilia, noticeable changes in nuclei, in particular, their wrinkling (karyopyknosis), and increased basophilia), which ends in lymphocyte necrosis.

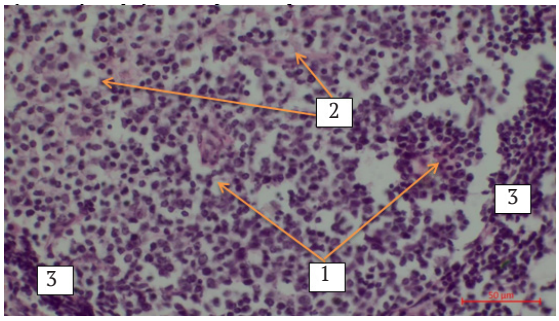

Figure 7. Reactive centres in the lymph follicle as a result of the reaction of lymphocytes to foreign antigens (haematoxylin and eosin staining)

Notes: *1 – initial stages of development of primary nodules from lymphocytes; 2 – reticulocytes under the influence of mycobacterial exotoxins; 3 – periphery of the reactive centre*

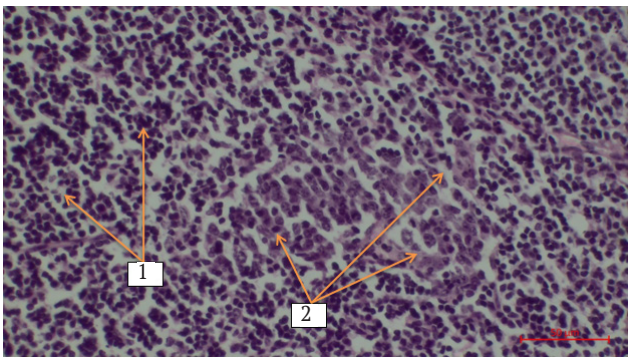

Figure 8. Reactive centres in the lymphatic follicle as a result of lymphocyte reaction to foreign antigens (haematoxylin and eosin staining)

Notes: *1 – clusters of normal lymphocytes; 2 – degraded lymphocytes with severe karyopycnosis and karyorexia under the influence of mycobacterial exotoxins in the process of primary nodule development*

Cellular detritus is developed at the site of lymphocyte death (Fig. 9.1), and a pronounced primary nodule is established, surrounded by a significant layer of lymphocytes (Fig. 9.2).

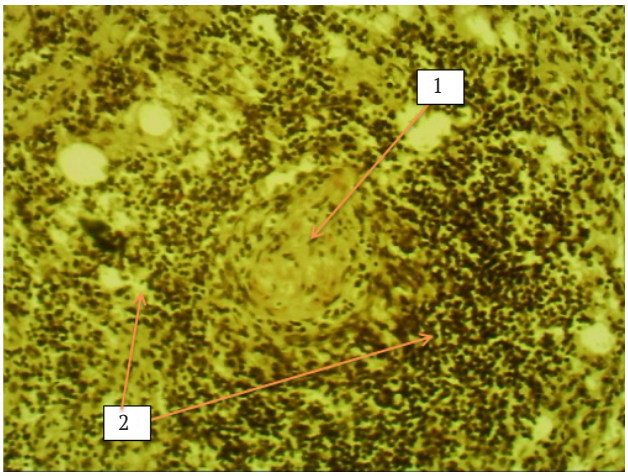

Figure 9. Development of nodules in NTBM-infected lymph nodes at later stages of the infectious process (hematoxylin and eosin staining. Magnification ×400)

Notes: *1 – cellular detritus in the centre of the nodule; 2 – accumulation of lymphoid elements around the detritus*

Similar morphological changes were found in histological sections of suprarenal lymph nodes (Fig. 10), where the development of primary nodules with cellular detritus in the centre (Fig. 10.1), a slight peripheral accumulation of lymphocytes (Fig. 10.2) and the establishment of a visible capsule of epithelioid elements (Fig. 10.3) were noted.

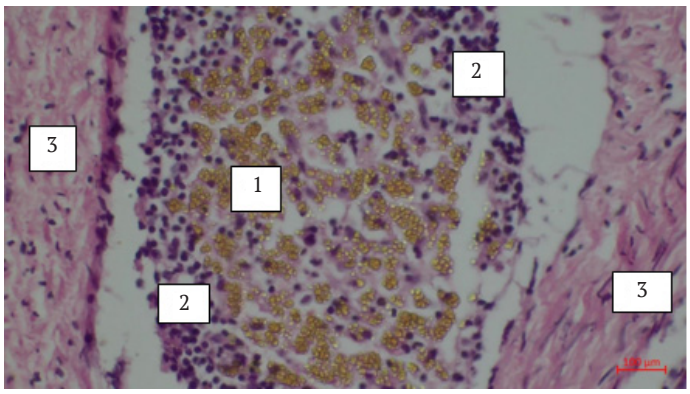

Figure 10. Development of nodules in NTMB-infected lymph nodes at later stages of the infectious process (haematoxylin-eosin staining)

Notes: *1 – cellular detritus in the centre of the nodule; 2 – accumulation of lymphoid elements around the detritus; 3 – formation of a capsule from epithelioid cells*

In no case did the authors detect calcification of these capsules, as is the case with tuberculous mycobacteria (*M. bovis* or *M. tuberculosis*). Evidently, such epithelioid strands develop complete or incomplete connective tissue capsules, merging, displacing or replacing the primary nodules formed in the reactive centres and, subsequently, the lymphoid follicles themselves and, ultimately, the cortical zone (Fig. 1.2).

Morphologically, this process is accompanied by the proliferation of epithelioid tissue, which leads to atrophy of the lymphoid tissue of the lymph nodes (Fig. 11.1). In cows of 7-9 years of age, more pronounced atrophy of the cortical layer of the lymph nodes was observed compared to this pathology in younger cows. It is probable that the longer the exposure of lymphoid tissue to NTMB pathogenicity factors, the more significant the atrophy of the cortical zone of the latter.

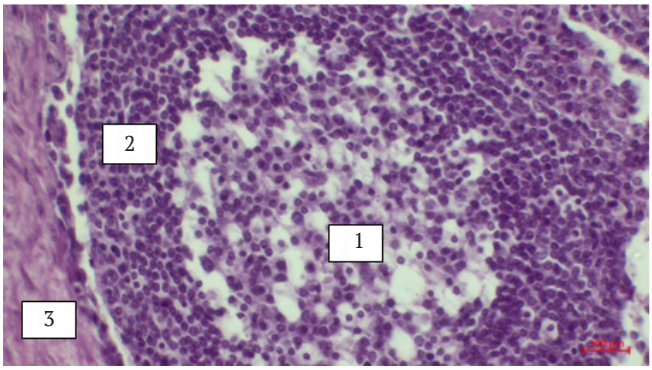

Figure 11. Development of nodules in lymph nodes infected with NTMB

Notes: *1 – necrosis of lymphocytes in the centre of the nodule, fragmentation of nuclei and condensation of chromatin, loss of whole areas of dead cells; 2 – a zone of lymphocytes around detritus; 3 – capsule of epithelioid cells*

The detected pathohistomorphological changes in the lymph nodes and in the submucosal layer of the large intestine indicate an active infectious process in the body of cows caused by NTMB. Although NTMBs were isolated from all lymph nodes taken for bacteriological examination (Boyko *et al.,* 2020), pathological changes were detected only in the supraclavicular, mediastinal, mesenteric, occasionally in the submandibular and pharyngeal lymph nodes and very rarely in the anterior and posterior phrenic and knee lymph nodes. These changes, along with cortical haemorrhages, can be detected visually when the nodes are sagittally incised. Developed primary nodules, which are noted during the histological examination, remain invisible visually and are not detected by palpation.

It can be assumed that primary nodules arise as a result of a powerful, both in terms of quantity and duration, exposure to antigenic irritation of the immune system, in particular the lymph nodes. As a result, cellular reactions occurring in the reactive zones lead to the necrosis of lymphocytes and their replacement by epithelioid cells (Figs. 9-12), which penetrate the cortical layer in the form of strands, starting from the cerebral layer, displacing lymphoid tissue and causing atrophy of the latter.

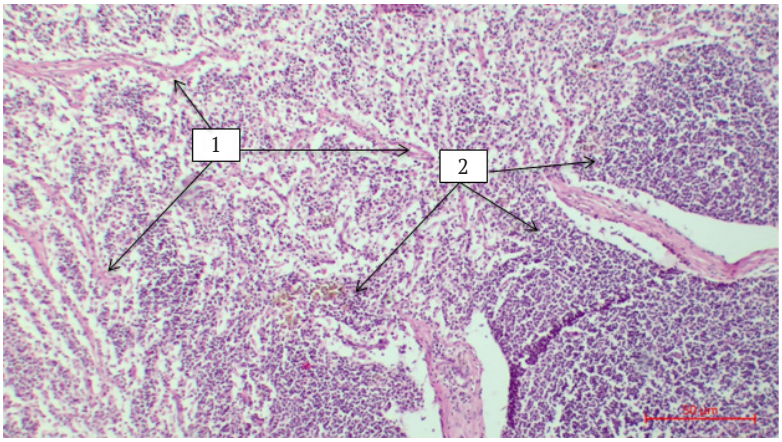

Figure 12. Replacement of lymphoid tissue with epithelioid elements in lymph nodes infected with NTMB as a result of the infectious process (hematoxylin and eosin staining)

Notes: *1 – cords of epithelioid cells; 2 – remnants of lymphoid tissue*

The establishment of granulomas in the lymph nodes (Fig. 3 A) confirms the prolonged effect of mycobacterial toxins on the immune system of animals, which leads to the development of a specific granulomatous inflammation. The mechanism of its development is based on primary perivascular infiltration by CD4+ T-lymphocytes, which are gradually (within 2-3 weeks) replaced by macrophages, and the latter, due to their activation by cytokines, increase in size and resemble squamous epithelium in shape, their cytoplasm becomes eosinophilic, and they gradually transform into epithelioid cells. Some cytokines, such as IFN-γ, acting on transformed epithelioid cells, transform them into multinucleated giant cells (Kumar *et al.,* 2019). Foci of mycobacterial penetration and proliferation lead to the accumulation of epithelioid cells, including giant cells. And these clusters, in turn, are surrounded by a thick shaft of lymphocytes, resulting in the development of granulomas (Fig. 13).

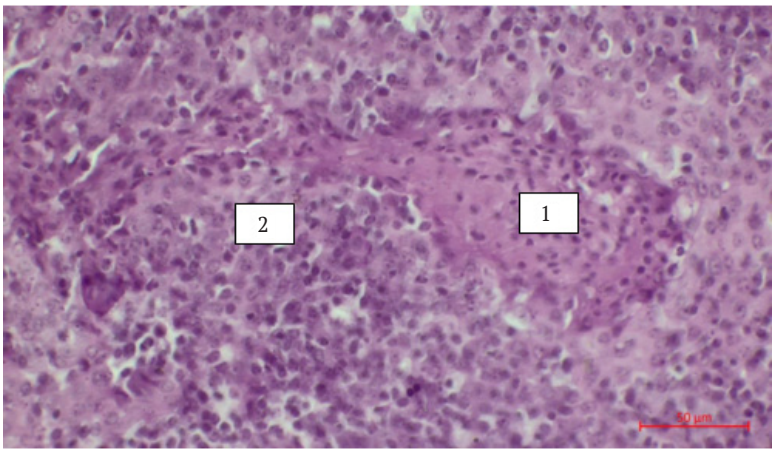

Figure 13. Granuloma development (haematoxylin and eosin staining)
Notes: *1 – clusters of epithelioid cells surrounded by a thick shaft of lymphocytes (2)*

T-cell-mediated inflammation underlies the mechanism of tissue damage in many autoimmune diseases or those caused by uncontrolled reactions to commensal bacteria or atypical acid-fast mycobacteria when they are constantly and in large volumes ingested by animals (inflammatory bowel disease in humans (Kumar *et al.,* 2019). In the present research, the source of NTBM in the organs and tissues of cows was chronic local interstitial colitis (Figs. 4, 5).

The pathological changes presented in Figs. 7-13, pathomorphological changes occur at the cellular and tissue levels and are characterised by irreversibility. These pictures demonstrate the development of primary nodules from lymphocytes and reticular tissue, lymphocyte degradation – the cytoplasm swells, its eosinophilia increases; changes in the nuclei, resulting in their wrinkling (karyopyknosis), which in turn is manifested by increased basophilia, and karyorexis (nucleus fragmentation), which ends with karyolysis of the nuclei and necrosis of lymphocytes. The affected lymphoid tissue is replaced by epithelioid tissue, which leads to atrophy of the lymph node parenchyma.

Evidently, the body's sensitisation to NTMB antigens begins at the stage of primary nodule development in the reactive centres of the lymph nodes and is permanently maintained by each subsequent ingestion of a fresh portion of NTMB from the environment.

Finally, the possibility of permanent ingestion of NTMBs from the digestive tract, where mycobacteria can be in the entobiotic phase of existence, colonising the submucosal layer of the large intestine wall, cannot be excluded (Boyko *et al.,* 2020a; Boyko *et al.,* 2020b). However, it is assumed that this mechanism of maintaining the sensitisation of the cow's organism is not decisive, as then the permanence of paraallergic reactions remains unexplained.

It can be argued that the permanence of paraallergic reactions in animals is a consequence of a significant amount of NTMB entering the body of cows over a long period (according to observations, at least 2-3 months) from the outside (alimentary or aerogenic).

Exploring the epizootological features of cow mycobacteriosis in a particular farm, it was found that the number of cows reacting to both allergens was significantly (2-3 times) higher

in the allergic test after winter-stall housing compared to that in summer-exercise housing. It proves that keeping animals indoors for a long period (more than 2-3 months) leads to constant ingestion of NTMBs, which cause the pathomorphological changes identified and described and manifest themselves as a permanent allergy to tuberculin and NTM.

According to the authors, a significant reduction in the antigenic load on the body of NTMB cows would reduce the infectious process in the body of infected animals and, thus, sensitise the body of cows to allergens of atypical mycobacteria. The assumptions made are fully consistent with the findings published earlier by some researchers (Falkinham, 2013). They argue that any changes in human health or animal husbandry conditions could lead to the emergence of new infections through NTMB as a result of the widespread distribution of these mycobacteria in most ecological niches (water, soil, sphagnum and wildlife) (Schroder *et al.,* 1992).

For this purpose, it is necessary to explore more thoroughly the ecology of NTMBs on the territory of the dairy farm. It will allow for a more targeted and effective implementation of several preventive measures aimed primarily at breaking the epizootic chain by reducing NTMB on the premises and the farm. It will have an objective impact on the welfare of the dairy herd and reduce or even eliminate the risk of spreading NTMB through dairy products, which will be of great medical and epidemiological importance for the prevention of human mycobacteriosis.

Conclusions

According to the results obtained, it was proved that the epizootic process in cattle herds is caused by the infection of animals with acid-fast mycobacteria. This phenomenon is quite common and therefore should be constantly monitored by qualified specialists of the state veterinary service.

Nontuberculous mycobacteria colonise mainly the digestive and respiratory organs, which indicates their alimentary and aerogenic routes of entry into the animal body. They linger for the longest time in the regional lymph nodes of these organs, where they cause profound and often irreversible pathological changes at the cellular and tissue levels, which were identified through detailed pathological and histological examinations. The pathomorphological changes in the lymph nodes and large intestine of the reacting animals can be used to determine the presence of an intense infectious process caused by NTMB in their bodies and the functioning of the epizootic process – mycobacteriosis – in the dairy cow herd. Thus, reactive animals should be considered as an active source of NTBM, which is important both epizootologically and epidemiologically.

More detailed research on the species composition of NTMBs infecting animals and the ecology of NTMBs identified on the farm and their potential pathogenic properties will be of both economic and epidemiological and medical epidemiological importance. Considering the significant role of NTBMs in human infectious pathology, each case of establishing the functioning of the epizootic process of mycobacteriosis in herds of productive animals should be considered as a source of potential biohazards to human health in the food chain. Therefore, further research will focus on an in-depth study of other aspects of the mycobacteriosis infection process, the ecology of NTMBs on the territory of a dairy farm, and ways to minimise the biohazards of human infection with NTMBs that cause dangerous meat and dairy food products.

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None.

Conflict of Interest

None.

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Патоморфологічні зміни в органах периферичної імунної системи за мікобактеріозів великої рогатої худоби

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Анотація. Проведення планових алергічних досліджень великої рогатої худоби симультанною пробою в господарствах благополучних щодо туберкульозу є необхідним для підтвердження стадії епізоотичного процесу. Мета роботи – дослідити патоморфологічні зміни у корів за виявлення парааллергічних реакцій під час діагностичного дослідження на туберкульоз. Діагностику туберкульозу в тварин здійснювали комплексно з використанням усіх передбачених інструкцією методів щодо боротьби з туберкульозом – епізоотологічного, клінічного, алергічного, патологоанатомічного, гістоморфологічного, бактеріологічного і біологічного. Завдяки цьому вдалося встановити, що реакції на туберкулін у корів мали парааллергічну природу, викликані нетуберкульозними мікобактеріями або, так званими, атиповими мікобактеріями. Встановлено, що у корів, які позитивно реагували на туберкулін і на алерген атипових мікобактерій та були відправлені на забій з діагностичною метою, не виявлено патологоанатомічних змін, характерних для туберкульозу. Проте, у багатьох лімфатичних вузлах, зокрема медіастинальних, мезентеріальних, заглоткових

і нижньощелепних, відмічали патологічні зміни (грануломатозні вогнища, атрофію лімфоїдної і розростання епітеліоїдної тканин, гіперплазію різної інтенсивності та характеру, крапчасті крововиливи у кірковій зоні), які вказують на активний інфекційний процес в організмі тварин, спричинений нетуберкульозними мікобактеріями. Водночас при проведенні гістоморфологічних досліджень у лімфатичних вузлах відзначали низку змін: формування первинних вузликів із лімфоцитів та ретикулярної тканини у реактивних центрах, деградацію та некроз лімфоцитів, утворення клітинного детриту у центрі вузликів та капсул із епітеліоїдних елементів навколо вузликів тощо, за якими можна підтвердити закономірності розвитку патології і, опосередковано, – механізм сенсибілізації організму корів, а отже і природу парааллергічних реакції у тварин. Отримані результати мають важливе значення для своєчасного виявлення та ідентифікації на гістоморфологічному рівні комплексу змін в органах і тканинах корів за проникнення нетуберкульозних мікобактерій, що є запорукою успішної діагностики первинних вогнищ мікобактеріозів та ефективної їх профілактики

Ключові слова: туберкульоз; туберкулін; антиген атипових мікобактерій; інфекційний процес; атипові мікобактерії; алергічні реакції; діагностика

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Antibiotic resistance of microorganisms isolated from cows with subclinical mastitis

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Abstract. Cow mastitis is a polyetiological disease involving pathogenic microorganisms. Antibiotics are used to treat sick animals, which contributes to the development of resistant clones of bacteria, and consumption of the corresponding milk leads to the spread of antibiotic-resistant microorganisms. The purpose of the work is to determine strains of antibiotic-resistant bacteria in the milk of cows suffering from subclinical mastitis. Bacteriological studies of mammary gland secretion samples were performed by inoculating the sediment obtained after centrifugation of the examined material on an agarised nutrient medium (with 5% ram erythrocytes). The study of antimicrobial sensitivity was performed by the disk diffusion method. It was established that pathogens with multiple resistance to antibiotics circulate among cows suffering from mastitis on the farm, which indicates their indiscriminate use in animal therapy schemes, in particular mastitis. It was found that the overall level of phenotypic resistance to antimicrobial agents of the examined isolates was within 19-33% (in *Staphylococcus spp*. – 19.9%, in *E. coli* – 33.2%, in *Enterococcus spp*. – 32.5%). The analysis of the results allowed assigning the culture study to the category of multiresistant: 31.6% of cultures of *Staphylococcus spp*. demonstrated resistance to five groups of antibiotics; 54.5% of Escherichia cultures – up to five groups of antibiotics; 37.5% of cultures of *Enterococcus spp*. resistant to three groups of antibiotics. It was established that 100% of the studied cultures of *Staphylococcus spp*. are sensitive to netilmicin, sparfloxacin, gatifloxacin and clarithromycin. Therewith, 100% of the examined cultures of *Enterococcus spp*. sensitive to gentamicin, norfloxacin, and nitrofurantoin, and 100% of the examined *E. coli* cultures were sensitive to cephalothin, meropenem, gentamicin, tobramycin, amikacin, nitroxoline, and gatifloxacin. Thus, dairy cows suffering from subclinical mastitis are a source of bacteria with multiple resistance to antibiotics and carry risks for consumers due to the consumption of dairy products contaminated with pathogens of zoonotic diseases, which requires proper control by veterinary service specialists

Keywords: antimicrobial drugs; disk diffusion; antibiotic-resistant bacteria; antibiotic therapydairy cows; milk

Introduction

One of the key objectives of the animal husbandry industry is to ensure the demand of consumers with safe dairy products of appropriate quality, and the basis for their production is, accordingly, raw milk that corresponds to the requirements of current standards.

In Ukraine, the requirements for the quality of raw milk are harmonised with international standards, which define the microbiological criteria for the quality of raw milk: $\leqslant 1.0 \times 10^5$ – for milk of the extra grade; $\leqslant 3.0 \times 10^5$ for high-quality milk; $\leqslant 5.0 \times 10^5$ for milk of the first grade (DSTU 3662:2018, 2019).

However, in many cases, the sustainable production of livestock products is hindered by diseases, in particular mastitis in lactating cows. Mastitis in cows is an inflammation of the mammary gland under the action of, as a rule, a complex etiological factor with a prominent role of microbial agents (Amer *et al.,* 2018). It is important to consider that the course of mastitis, as indicated by the authors (Dittrich *et al.,* 2019, Blackmon *et al.,* 2023), is associated with a deterioration of the animal's welfare – painful sensations (redness, pain on palpation, swelling and hardening of the skin of the udder) and

deterioration of well-being (fever, anorexia, depression, lethargy, etc.).

The development of the disease is accompanied by significant losses of productivity and a decrease in milk quality indicators (Romero *et al.,* 2018). Significant economic losses are caused both by a decrease in productivity and by low reproduction rates and possible premature culling of cows (Cheng & Han, 2020).

According to many authors, the subclinical form of mastitis is most often registered among lactating cows around the world (Cheng & Han, 2020; Danchuk *et al.,* 2021); (Sharun *et al.,* 2021; Pascu *et al.,* 2022; Saeed *et al.,* 2022), which is accompanied by an increase in the content of somatic cells and changes in its physical and chemical qualities, in particular, the fatty acid composition.

Among the etiological factors of mastitis in cows, microorganisms play an important role, thus, some authors indicate that more than 140 types of microorganisms can be causative agents of mastitis (Goulart & Mellata, 2022). However, the development of mastitis is most often caused by Staphylococcus aureus, coagulase-negative Staphylococcus (non-aureus), *Escherichia coli*, *Corynebacterium bovis*, *Streptococcus uberis* and *Streptococcus dysgalactiae*, which are considered the most common pathogens of the mammary gland (Heikkilä *et al.,* 2018).

As the authors note (Heikkilä *et al.,* 2018; Goulart & Mellata, 2022), economic losses due to mastitis in cows in the USA are in the range of $2 billion, with losses from each case estimated at $326. The course of clinical mastitis caused by pathogenic Escherichia coli causes Holstein cows to decrease daily milk yield by almost 15%, which is considered an average milk loss of approximately 200 litres per cow during the 305-day lactation period.

As a result of the analysis of losses during milk production in Finland (Heikkilä *et al.,* 2018) it was established that mastitis caused by *Escherichia coli* caused the loss of 10.6% of milk from a 305-day milking per day (~3.5 kg/day); lack of milk as a result of mastitis caused by *Staphylococcus aureus* was 7.1% (2.3 kg/day); mammary gland disease caused by *Streptococcus uberis* and *Streptococcus dysgalactiae* led to a shortage of milk in the range of 3.7% (1.2 kg/day) – 6.6% (2.1 kg/day); losses in mastitis caused by *Corynebacterium bovis* amounted to 7.4% (2.4 kg/day); losses in mastitis caused by *Staphylococcus* non-aureus – 5.7% (1.8 kg/day). The authors conclude that systematic measures to prevent and combat mastitis lead to a significant increase in the efficiency of dairy farms. Among the measures designed to combat the spread of mastitis, a particular place is given to specific prevention and immunotherapy (Vasileiou *et al.,* 2019; Chobyrka *et al.,* 2020; Rozos *et al.,* 2022).

Notably, in addition to the risks of reducing the economic efficiency of dairy farms and losses of livestock products, mastitis carries risks to human health – both in an individual aspect due to the risks of consuming dairy products contaminated with zoonotic microorganisms with a high pathogenic potential (Sugrue *et al.,* 2019; Maity & Ambatipudi, 2020), and public health in general – potential spread with milk and dairy products of antibiotics residues and antibiotics resistant to microorganisms, the development of which is caused by the indiscriminate use of antibiotics for the treatment of mastitis (Boireau *et al.,* 2018; Singh *et al.,* 2018), which are critically important for humans, for example – cephalosporins and fluoroquinolones, colistin, tetracycline and macrolides (McEwen & Collignon, 2018).

The purpose of the work was to explore the signs of resistance to antibiotics in microorganisms isolated from cows with subclinical mastitis.

Materials and Methods

The diagnosis of subclinical mastitis was made based on the results of milk studies on the farm from the farms of the Poltava regions using the California mastitis test (Avita test). For microbiological research, udder secretion samples were taken from 23 cows with subclinical mastitis. The study complied with ARRIVE recommendations and was conducted without violating the guidelines of EU Directive 2010/63/EU on the protection of animals used for scientific purposes (2010).

Culture media were prepared and controlled according to ISO 11133:2014 "Microbiology of food, animal feeding stuffs and water" (2014). Preparation, production, storage and performance testing of culture media. The nutrient media, commercial tests, and discs with antimicrobial drugs manufactured by HiMedia were used in the study.

Bacteriological studies of mammary gland secretion samples were conducted by sowing the sediment obtained after centrifugation of the examined material on an agarised nutrient medium (with 5% ram erythrocytes and 0.05% esculin), which ensures the vegetation of most microorganisms that cause the development of mastitis. The isolated microorganisms were subjected to further differentiation according to cultural-morphological, tinctorial signs and the ability of bacteria to metabolise substrates. In addition, for the identification of isolated bacterial cultures, the relevant recommendations presented in ISO 16649-2:2015 "Microbiology of food and animal feeding stuffs". Horizontal method for the enumeration of -glucuronidase-positive Escherichia coli; SSU 8534: 2015 "Food products. Method for detection and determination of Enterococci" (2017).; ISO 6888-1:2021 (2021) "Microbiology of food and animal feeding stuffs" – Horizontal method for the enumeration of coagulase-positive staphylococci.

Antimicrobial susceptibility testing was performed by the disc diffusion method (considering some provisions of EUCAST, 2021). The results of the study were recorded and interpreted using an automatic colony counter Scan® 500 manufactured by INTERSCI.

Results and Discussion

This research presents the results of the study of selected cultures of zoonotic microorganisms from milk samples obtained from cows with signs of subclinical mastitis. As a result of the study, 23 samples of udder secretion samples were isolated: 19 cultures of *Staphilococcus spp.* (13 coagulase-positive staphylococci (CoPS) and 6 coagulase-negative staphylococci (CoNS), 8 cultures of *Enterococcus spp.* (*Enterococcus faecalis* and *Enterococcus faecium*) and 11 cultures of *Escherichia coli*.

The results of sensitivity/resistance studies of isolates of *Staphylococcus spp*. are presented in Table 1.

Table 1. The sensitivity of *Staphylococcus spp.* isolates from milk samples to antibacterial drugs (n=19)

Antibiotics	Cultures			ATU, mm
	S (n/%)	R (n/%)	ATU (n/%)	
Penicillins				
Benzylpenicillin	9/47.4%	10/52.6%	0	26/26
Ampicillin	7/36.8%	12/63.2%	0	18/18
Oxacillin	3/15.8%	13/68.4%	3/15.8%	17/18

Table 1. Continued

Antibiotics	Cultures			ATU, mm
	S (n/%)	R (n/%)	ATU (n/%)	
Aminoglycosides				
Gentamicin (I)	17/89.5%	2/10.5%	0	22/22
Kanamycin	14/73.7%	3/15.8%	2/10.5%	13/18
Tobramycin (II)	18/94.7%	1/5.3%	0	22/22
Netilmycin	19/100%	0	0	12/15
Amikacin (III)	16/84.2%	3/15.8%	0	22/22
Tetracyclines				
Tetracycline	11/57.9%	6/31.6%	2/10.5%	19/22
Doxycycline	13/68.4%	1/5.3%	5/26.3%	12/16
Quinolones				
Sparfloxacin	19/100%	0	0	15/19
Norfloxacin (II)	17/89.5%	1/5.3%	1/5.3%	17/17
Ciprofloxacin (II)	1/5.3%	5/26.3%	13/68.4%	24/50
Ofloxacin (II)	0	6/31.6%	13/68.4%	17/18
Levofloxacin (II)	0	4/21.1%	15/78.9%	24/50
Pefloxacin (II)	16/84.2%	0	3/15.8%	15/22
Gatifloxacin (IV)	19/100%	0	0	14/18
Lomefloxacin	16/84.2%	0	3/15.8%	18/22
Other antibiotics				
Nitrofurantoin	14/73.7%	5/26.3%	0	13/13
Chloramphenicol	18/94.7%	0	1/5.3%	18/18
Fusidic acid	12/63.2%	0	7/36.8%	24/24
Rifampicin	16/84.2%	1/5.3%	2/10.5%	23/26
Lincomycin	17/89.5%	1/5.3%	1/5.3%	17/21

Note: *"S" is sensitive; "R" – resistant; "ATU" – Area of Technical Uncertainty*
Source: *author's development*

The analysis of the obtained data demonstrated that among the examined staphylococci, 61.5% of the isolates were resistant to penicillins; resistant to aminoglycosides were: 15.7% of isolates – to amikacin and 5.2% – to tobramycin; resistant to tetracycline drugs was 31.6% – to tetracycline and 5.3% – to doxycycline; resistant to drugs of the quinolone group were found: 26.3% – to ciprofloxacin, 31.6% – to ofloxacin, 21.1% – to levofloxacin. 36.8% of isolates were resistant to fusidic acid, 26.3% to nitrofurantoin, 15.8% to lincomycin, and 5.3% to chloramphenicol. Thus, the circulation of staphylococci with multiple resistance to antimicrobial agents was detected, which indirectly indicates their indiscriminate use for antibacterial therapy of animals on this farm.

Results of sensitivity/resistance studies of isolates of *Enterococcus spp*. are presented in Table 2.

Table 2. The sensitivity of isolates of *Enterococcus spp.* from milk samples to antibacterial preparations (n=8)

Antibiotics	Cultures			ATU, mm
	S (n/%)	R (n/%)	ATU (n/%)	
Penicillins				
Benzylpenicillin	3/37.5%	4/50%	1/12.5%	14/15
Ampicillin	5/62.5%	2/25%	1/12.5%	8/10
Carbapenems				
Imipenem	0	6/75%	2/25%	21/50
Aminoglycosides				
Gentamicin (I)	8/100%	0	0	6/10
Streptomycin	6/75%	1/12.5%	1/12.5%	6/10
Tetracyclines				
Tetracycline	4/50%	3/37.5%	1/12.5%	14/19
Doxycycline	4/50%	2/25%	2/25%	12/16
Quinolones				
Norfloxacin (II)	8/100%	0	0	12/12
Ciprofloxacin (II)	6/75%	2/25%	0	15/15
Levofloxacin (II)	6/75%	2/25%	0	15/5
Gatifloxacin (IV)	3/37.5%	0	5/62.5%	14/18
Other antibiotics				
Nitrofurantoin	8/100%	0	0	15/15
Chloramphenicol	6/75%	1/12.5%	1/12.5%	12/18
Vancomycin	5/62.5%	3/37.5%	0	12/12
Erythromycin	0	2/25%	6/75%	13/23

Note: *"S" – sensitive; "R" – resistant; "ATU" – Area of Technical Uncertainty*
Source: *author's development*

The results of determining the antibiotic profile of Enterococcus isolates identify resistance to antibiotics from the penicillin group: 25% of isolates to ampicillin and 50% to benzylpenicillin; 75% of the examined isolates were resistant to imipenem; resistance to quinolones was found in: 25% of isolates to ciprofloxacin and levofloxacin, respectively; resistant to drugs of the tetracycline group, therewith, to tetracycline – 37.5%, to doxycycline – 25%; resistance to chloramphenicol was found in 25% of the examined isolates; 37.5% of enterococci were resistant to vancomycin, 25% were resistant to erythromycin.

Table 3. Sensitivity of *E. coli* isolates from milk samples to antibacterial drugs (n=11)

Antibiotics	Cultures			ATU, mm
	S (n/%)	R (n/%)	ATU (n/%)	
Penicillins				
Ampicillin	4/36.4%	7/63.6%	0	14/14
Ampicillin/sulbactam	10/90.9%	1/9.1%	0	14/14

Table 3. Continued

Antibiotics	Cultures			ATU, mm
	S (n/%)	R (n/%)	ATU (n/%)	
Amoxicillin-clavulanic acid	0	11/100%	0	19/19
Ticarcillin	5/45.5%	4/36.4%	2/18.2%	17/20
Ticarcillin / clavulanic acid	0	6/54.5%	5/45.5%	20/23
Piperacillin	7/63.6%	0	4/36.4%	17/20
Cephalosporins				
Cefalotin (I)	11/100%	0	0	14/18
Cephalexin (I)	11/100%	0	0	14/14
Cefazolin (I)	10/90.9%	1/9.1%	0	1418
Cefaclor (II)	2/18.2%	5/45.5%	4/36.4%	14/18
Cefuroxime (II)	0	11/100%	0	
Cefamandole (II)	7/63.6%	1/9.1%	3/27.3%	14/18
Cefixim (III)	9/81.2%	2/18.2%	0	17/17
Cefoperazone (III)	8/72.3%	0	3/27.3%	15/21
Cefotaxim (III)	10/90.9%	1/9.1%	0	17/20
Cefepim (IV)	0	10/90.9%	1/9.1%	24/27
Cefpodoxime (III)	11/100%	0	0	21/21
Carbapenems				
Meropenem	11/100%	0	0	16/22
Aminoglycosides				
Gentamicin (I)	11/100%	0	0	17/17
Kanamycin (I)	3/27.3%	0	8/72.3%	13/18
Tobramycin (II)	11/100%	0	0	17/17
Netilmicin	11/100%	0	0	12/15
Amikacin (III)	11/100%	0	0	18/18
Tetracyclines				
Tetracycline	0	11/100%	0	19/19
Doxycycline	1/9.1%	10/90.9%	0	12/16
Quinolones				
Nalidixic acid (I)	8/72.3%	0	3/27.3%	13/19
Nitroxoline (I)	11/100%	0	0	15/15
Ciprofloxacin (II)	10/90.9%	0	1/9.1%	22/25
Ofloxacin (II)	8/72.3%	1/9.1%	2/18.2%	22/24
Lomefloxacin (II)	10/90.9%	0	1/9.1%	18/22
Gatifloxacin (IV)	11/100%	0	0	14/18
Other antibiotics				
Chloramphenicol	11/100%	0	0	17/17

Note: *"S" – sensitive; "R" – resistant; "ATU" – Area of Technical Uncertainty*
Source: *author's development*

Among the studied *Escherichia coli* (Table 3), an average of 43.6% was resistant to Penicillins, 34.7% – from the group of Cephalosporins, 14.5% – from the Aminoglycosides group, 95.5% of *E. coli* cultures were resistant to Tetracyclines, 11.8% – were resistant to Quinolones.

According to the results of the study, it was found that pathogens with multiple resistance to antibiotics are circulating among the cows on this farm, which indicates their indiscriminate use for the antibacterial therapy of animals on this farm.

Thus, as a result of studies of cultures isolated from cows with signs of subclinical mastitis, it was established that 100% of the examined cultures of *Staphilococcus spp*. were sensitive to Netilmycin, Sparfloxacin, Gatifloxacin, Clarithromycin; 100% of the studied cultures of *Enterococcus spp.* were sensitive to Gentamicin, Norfloxacin, Nitrofurantoin; 100% of the studied *E. coli* cultures were sensitive to Cefalotin, Cephalexin, Cefpodoxime, Meropenem, Gentamicin, Tobramycin, Netilmicin, Amikacin, Nitroxoline, Gatifloxacin, Chloramphenicol, and resistant in 100% of cases to Amoxicillin-clavulanic acid, Cefuroxime (II), Tetracycline.

Many researchers have proven that mastitis is one of the most common diseases of dairy cattle, which causes economic losses due to a decrease in milk yield and poor milk quality. It is believed that the main cause of mastitis in cattle is the striking effect of microorganisms with a high pathogenic potential, which causes the development of inflammation of the udder. Measures designed to combat the spread of infectious mastitis have been identified (Bradley, 2002; Blowey & Edmondson, 2010), which include: identification and treatment of clinical cases, teat disinfection after milking, dry cow therapy (DCT), timely culling of cows when chronic cases of mastitis are registered, proper ongoing maintenance of milking equipment. However, practice demonstrates that the proposed actions are not sufficiently effective. Accordingly, there is an objective need to improve existing mastitis prevention and treatment measures, in particular, the improvement of etiotropic therapy schemes (Down *et al.,* 2016). The data obtained during the analysis of the epizootic situation with mastitis of cows in the studied farm indirectly testify to the imperfection of the implemented preventive and curative measures.

Currently, several microorganisms have been identified as causative agents of mastitis (Heikkilä *et al.,* 2018; Goulart & Mellata, 2022). But, more often, specialists identify representatives of the coccal microflora (*Staphylococcus spp., Streptococcus spp., Enterococcus spp*.) and a prominent representative of enterobacteria – *Escherichia coli*, as factors in the occurrence of mastitis, and, accordingly, in many cases, antibiotics are included in mastitis treatment regimens. The results of this research are consistent with the evidence of other authors regarding (Cheng & Han, 2020) the causative agents of cow mastitis.

Escherichia coli is a microorganism that can have in its arsenal a combination of pathogenicity factors (toxins, adhesins, invasins, capsule development, the ability to resist serum complement and iron absorption, resistance to antibiotics and the ability to colonise, the ability to produce biofilms), which provides the ability to persist in the dairy gland, causing recurrent mastitis (Kaper *et al.,* 2004; Fernandes *et al.,* 2011; Gomes *et al.,* 2016; Cheng & Han, 2020). *Enterococcus spp.* (primarily *E. faecalis and E. faecium*) – are frequently obligate microorganisms of organic litter in livestock premises, which have a set of pathogenic factors (toxicity, adhesiveness, antibiotic resistance, ability to form a biofilm, etc.), which provides microorganisms with the opportunity to colonise the epithelium of the mammary gland and persist

causing inflammation (Elhadidy & Zahran, 2014; Różańska *et al.,* 2019). Representatives of *Staphylococcus spp.*: coagulase-positive staphylococci are recognised causative agents of mastitis in a wide range of animals, and coagulase-negative staphylococci (*S. simulans, S. chromogens, S. hyicus, S. epidermis*) are a relatively "new" causative agent of mastitis – according to researchers (Vasudevan *et al.,* 2003; Taponen & Pyörälä, 2009; Simojoki *et al.,* 2012; Hamid *et al.,* 2017) diseases caused by them have a less severe course, causing mainly the subclinical form of mastitis and have a set of pathogenicity factors (adhesiveness, resistance to antibiotics, toxigenicity) and are environmental pathogens. Data obtained by the authors in previous years (Ushkalov *et al.,* 2021, Vishovan, *et al.,* 2021) indicate the presence of a high pathogenic potential in potential mastitis pathogens and are consistent with the cited authors.

Thus, one of the important factors in the pathogenicity of mastitis pathogens is resistance to antibiotics. It is known that improper use of antibacterial therapy contributes to the development of antibiotic-resistant clones of microorganisms. Milk obtained from cows suffering from subclinical mastitis potentially poses a threat to human health, as it can be a source of zoonotic pathogens for a long time (asymptomatic course of the disease), causing diseases of the skin, upper respiratory tract, etc. (Schamberger *et al.,* 2004; Maity & Ambatipudi, 2020). In this regard, it is the problem of subclinical mastitis that needs particular attention since bacterial etiological factors have the opportunity for a long time (before the diagnosis and the beginning of therapeutic measures) to pollute the environment, including livestock products and have the potential to spread to people (such as service personnel and on product consumers). Thus, there are objective risks of the spread of zoonotic microorganisms with high pathogenic potential, in particular with multiple resistance to antibiotics in the human population (Cheng & Han, 2020; Maity & Ambatipudi, 2020; Goulart & Mellata, 2022).

In the presented research, sensitivity to antibiotics in cultures of *Staphylococcus spp., Escherichia coli, Enterococcus spp.* was explored within a small sample size, isolated from udder secretion samples of animals suffering from subclinical mastitis on a commodity farm.

It was established that 100% of the examined cultures of *Staphilococcus spp*. were sensitive to Netilmycin, Sparfloxacin, Gatifloxacin, Clarithromycin; 100% of the studied cultures of *Enterococcus spp.* were sensitive to Gentamicin, Norfloxacin, Nitrofurantoin; 100% of *E. coli* cultures tested were sensitive to Cefalotin, Cephalexin, Cefpodoxime, Meropenem, Gentamicin, Tobramycin, Netilmicin, Amikacin, Nitroxoline, Gatifloxacin, Chloramphenicol.

Analysis of the obtained data presented that the overall level of phenotypic resistance of the examined isolates was within 19-33% (19.9% was found among *Staphylococcus spp*., 33.2% among *E. coli*, 32.5% among *Enterococcus spp*.). When determining sensitivity to antibiotics, EUCAST recommends determining both indicators of sensitivity and resistance and the zone of technical uncertainty – "Area of Technical Uncertainty". Accordingly, within the "Area of Technical Uncertainty" there were 6.36% of *E. coli* cultures, 12.8% – *Staphylococcus spp.,* 16.3% – *Enterococcus spp*. Thus, the level of resistance among bacterial populations associated with subclinical mastitis may have the corresponding potential for growth.

Further, evaluating the obtained data, an attempt was made to characterise the examined cultures of microorganisms according to the degree of resistance to antibiotics. An international group of experts (Magiorakos *et al.,* 2012) under the auspices of the European Centre for Disease Prevention and Control (ECDC) and the Centres for Disease Control and Prevention

(CDC) of the USA proposed a special terminology to describe acquired resistance profiles. The following resistance profiles are defined: multidrug resistance (MDR) – acquired lack of sensitivity to at least one agent in three or more categories of antimicrobial drugs; extensive drug resistance (XDR) – lack of sensitivity to at least one drug in all but two or fewer categories of antimicrobial drugs (ie, bacterial isolates remain sensitive to only one or two categories); pan-resistance (PDR) – defined as insensitivity to all agents in all categories of antimicrobials. The analysis of the results (Tables 1-3) allowed assigning the study of the culture to the category of MDR. Among the group of *Enterococcus spp*. 37.5% of cultures were resistant to three groups of antibiotics, 25% of cultures were resistant to two groups of antibiotics. Among Staphylococcus spp. 31.6% of cultures were resistant to five groups of antibiotic groups, 5.3% of cultures were resistant to four groups. Among *E. coli*, 54.5% of cultures were resistant to five groups of antibiotics, 18.2% of cultures were resistant to four groups of antibiotics.

Considering the small size of the sample, the presented results are indicative and will determine the field of further research on the study of pathogenicity (phenotypic and genotypic determinants) and virulence in microorganisms associated with subclinical mastitis.

Conclusions

It was established that cultures of *Staphylococcus spp*. isolated from milk from cows with signs of subclinical mastitis showed sensitivity to Netilmycin, Sparfloxacin, Gatifloxacin, Clarithromycin in 100% of cases; cultures of *Enterococcus spp*. in 100% of cases were sensitive to Gentamicin, Norfloxacin, Nitrofurantoin; *E. coli* cultures showed sensitivity to Cefalotin, Cephalexin, Cefpodoxime, Meropenem, Gentamicin, Tobramycin, Netilmicin, Amikacin, Nitroxoline, Gatifloxacin, Chloramphenicol in 100% of cases. The level of resistance of the examined isolates was in the range of 19-33%, the analysis of the results allowed classifying the study isolates into the MDR category. Thus, milk from cows with subclinical mastitis is a risk factor for the spread of antibiotic-resistant bacterial clones. Measures at the level of individual livestock farms designed to reduce the risks of the spread of antibiotic-resistant microorganisms, in particular, etiologically significant in mastitis in cows, focus on ensuring the well-being of animals, increasing the level of biosecurity and introducing the technology of production of livestock products, elements of permanent monitoring of the condition of the udder, timely diagnosis of mastitis, if necessary – laboratory and it is possible to use means of extemporaneous formulation and autogenous immunobiological drugs – as an alternative to antimicrobial agents. There is reasonable evidence that preventive measures using autogenous vaccines, sera and globulins can be of significant importance for reducing the level of use of antimicrobial substances in the production of livestock products, which will contribute to the disruption of the food route of transmission of resistant populations of bacteria and metabolites of antimicrobial drugs. It is believed that autogenous immunobiological drugs can be used both for specific prevention and for the treatment of persistent infections.

Further research can be designed to establish a collection of strains of microorganisms – the causative agents of mastitis, studying the signs of pathogenicity in them and their possible use for the production of an autovaccine for the prevention of this dangerous disease.

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Conflict of Interest

None.

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Антибіотикостійкість мікроорганізмів, виділених від корів, хворих на субклінічний мастит

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Анотація. Мастит корів – поліетіологічне захворювання за участю патогенних мікроорганізмів. Для лікування хворих тварин використовуються антибіотики, що сприяє утворенню стійких клонів бактерій, а споживання відповідного молока призводить до поширення стійких до антибіотиків мікроорганізмів. Мета роботи – визначити штами антибіотикорезистентних бактерій у молоці корів, хворих на субклінічний мастит. Бактеріологічні дослідження проб секрету молочної залози проводили шляхом посіву осаду, отриманого після центрифугування досліджуваного матеріалу, на агаризоване живильне середовище (з 5 % еритроцитів барана). Дослідження антимікробної чутливості здійснювали методом дискової дифузії. Встановлено, що серед хворих на мастит корів у господарстві циркулюють збудники з множинною стійкістю до антибіотиків, що вказує на їх невибіркове застосування в схемах терапії тварин, зокрема маститу. Виявлено, що загальний рівень фенотипової стійкості до антимікробних засобів досліджуваних ізолятів знаходився у межах 19-33 % (у *Staphylococcus spp.* – 19.9 %, у *E. coli* – 33.2 %, у *Enterococcus spp*. – 32.5 %). Аналіз

результатів дав можливість віднести досліджені культури до категорії мультирезистентних: 31.6 % культур *Staphylococcus spp*. виявляли стійкість до п'яти груп антибіотиків; 54.5 % культур ешерихій – до п'яти груп антибіотиків; 37.5 % культур групи *Enterococcus spp.* стійкі до трьох груп антибіотиків. З'ясовано, що 100 % досліджених культур *Staphilococcus spp*. є чутливими до нетилміцину, спарфлоксацину, гатифлоксацину та кларитроміцину. При цьому, 100 % досліджених культур *Enterococcus spp*. чутливі до гентаміцину, норфлоксацину та нітрофурантоїну, а 100 % досліджуваних культур кишкової палички виявилися чутливими до цефалотину, меропенему, гентаміцину, тобраміцину, амікацину, нітроксоліну та гатифлоксацину. Таким чином, дійні корови, хворі на субклінічний мастит, є джерелом бактерій з множинною стійкістю до антибіотиків і несуть ризики для споживачів внаслідок вживання молочних продуктів, контамінованих збудниками зоонозних захворювань, що потребує належного контролю з боку фахівців ветеринарної служби

Ключові слова: антимікробні препарати; дискова дифузія; антибіотикорезистентні бактерії; антибіотикотерапія; дійні корови; молоко

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Determination of heart rate variability as an indicator of the influence of autonomic nervous system tone in cows

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Abstract. The relevance of the subject is the significance of exploring the role of the autonomic nervous system in regulating the cardiovascular system to improve the welfare of productive animals. Heart rate variability is a non-invasive research method that can be useful in exploring the health status of an animal and analysing its psychophysiological state in farm conditions. The purpose of the research – to determine the effect of the tone of autonomic nervous regulation on the cow's body, which is reflected in changes in the sympathovagal balance. Experimental research was conducted on Ukrainian Black-and-White dairy cows. To explore the variability of heart rate, an electrocardiograph was used, followed by the determination of the main indicators according to the Baevsky method, which included the determination of mode, mode amplitude, variation range, autonomic balance index, autonomic rhythm index and stress index. Based on the results of the study, three experimental groups of animals were established: normotonics, vagotonics, and sympathotonics. Considering the results obtained, cows, depending on the influence of the tone of the autonomic nervous system, have differences in the activity of the cardiovascular system. It will result in different responses to stress, which in turn will affect their productivity. Determination of heart rate variability can be one of the indispensable indicators in analysing the health of an animal on a dairy farm. This issue is a promising area of research, especially when exploring the metabolic processes of high-yield cows to improve productivity while maintaining the physiological state of the animal

Keywords: sympathovagal balance; normotonics; vagotonics; sympathotonics; animal health; cattle

Introduction

Many techniques have been developed to improve the welfare of modern dairy farms producing animal products. One of the most frequently asked questions is the impact of stress on animals. It is explained by the fact that cows are kept on farms in a relatively isolated environment, with no possibility of migration outside the paddock (Periyanayagi *et al.,* 2022). The animal's motility is stable and consistent with familiar places, surroundings and climatic conditions. When the environment in which a cow lives changes, stress occurs in its body under the influence of a new factor. Depending on individual characteristics, animals react differently to these changes (Kremer *et al.,* 2022). One of the key factors in the mechanism of response to this stress is changes in the autonomic nervous system. It is involved in the regulation of homeostasis along with other structures of the body through its departments (Mulkey & du Plessis, 2019; Jänig, 2022). Under the influence of the sympathetic nervous system, the activity of the cardiovascular system increases, the accumulation of nutrients in the bloodstream increases, and they are distributed to all organs and tissues of the animal's body. Due to this, the body has the necessary energy to ensure a proper response to the stressor (Bellato *et al.,* 2020; Weber *et al.,* 2021). The parasympathetic nervous system performs the opposite functions: it reduces the activity of the cardiovascular system and activates the processes of digestion and synthesis of nutrients to establish a reserve for immediate use (Gibbons, 2019; Benarroch, 2020).

Cardiovascular studies provide informative indicators that reflect the health and welfare of many animals. The researchers initially analysed heart rate to reflect the activity of the sympathetic and parasympathetic nervous systems. However, the results were mixed

(Quevedo *et al.,* 2019; Turini *et al.,* 2022). Changes in heart rate fluctuated greatly under the influence of physical and psychological factors. It could include a change in the animal's environment, pain, for example, as a result of medical procedures, or a stranger in the cows' location. To obtain more accurate data, they began to measure cardiac intervals based on cardiological and psychophysiological diagnostic methods. It allowed for a better interpretation of cardiac activity in terms of the activity of the autonomic nervous system. Heart rate intervals are frequently referred to as heart rate variability, which reflects the sympathetic-vagal balance. Considering this indicator, it allows for a better analysis of animal health, welfare, and psychophysiological state (Kovács *et al.,* 2019; Grelet *et al.,* 2022).

Electrocardiography is one of the most effective and non-invasive methods of exploring heart rate variability. During the recording of the heart's electrical potentials, fluctuations in the variability of successive heartbeat intervals can be recorded. Depending on the individual characteristics of the animal organism, the activity of the autonomic nervous system varies. It is reflected in an increase or decrease in the heart rate variability. These differences are explained by the cow's different perception of the environment and adaptation to the changes it encounters throughout its life. Most scientists, based on their research, emphasise the multidimensionality of individual animal characteristics (Hunter *et al.,* 2021; Kitajima *et al.,* 2022). Reactivity is one of the most common signs to measure the state of a cow's body. It is frequently replaced by behavioural reactivity in a person. Since each animal develops in a specific familiar environment, any change can cause stress (Emelyanova *et al.,* 2020). The reason for this may be a stranger in the cow's resting area or milking parlour. Depending on its reactivity, the animal may try to increase its distance to interact less with the stressor or, on the contrary, demonstrate interest and activity in the opposite area (La Maestra *et al.,* 2021; Devi *et al.,* 2022). When analysing the activity of the cardiovascular system, certain groups of animals can be identified based on the sympathetic-vagal balance. Normotonics will have a balanced equilibrium of both systems, sympathotonics – with a predominance of the sympathetic nervous system and vagotonics – with a greater influence of the parasympathetic nervous system. Accordingly, cows will have different responses to the factors (Gibbons, 2019; Cheshire *et al.,* 2020).

The purpose of the research – to determine the effect of the tone of autonomic nervous regulation on the body of cows, which is reflected in changes in sympathovagal balance.

Materials and Methods

The research was conducted based on the dairy farm of "Obriy" LLC, the breed of cows is Ukrainian black-and-white dairy. The research was conducted according to the ARRIVE guidelines for reporting experiments using live animals. 100 clinically healthy cows of 3-4 lactation were selected for the variation-pulse oximetry study. Electrocardiographic examination was performed using a single-channel electrocardiograph Heart Mirror IKO (Hungary, Innomed). An electrocardiograph – is a voltmeter or galvanometer that records changes in the electrical potential of the heart. The animal should be sufficiently rested before electrocardiography. The cow is then kept in a standing position. Electrocardiological gel was applied to the electrode attachment sites after pretreating the skin with alcohol. Three sites were used for the research. The first is located between 3-5 intercostal spaces on the left, behind the elbow. The electrode of the right hand was attached to it. The second one is located to the caudal 1/3 of the jugular sulcus, where

the left-hand electrode was placed. The third – in the withers of the animal, where a neutral lead was placed. During the research, a "crocodile" clamp was used. These clips have a sharp mouth with serrated ends to securely hold them in place on thick cattle skin. The toothed ends are very sharp, thus, they need to be dulled beforehand to avoid injuring the animal. During the recording of changes in the electrical potentials of the heart occurring during each cardiac cycle, the corresponding indicators were recorded by a galvanometer. The data was recorded using a stylus on cardiac examination paper. The stylus deflects according to the intensity of the heart's electrical activity (Jackson & Cockcroft, 2008). The tape pulling speed during cardiac signal recording was 50 mm/s. The results of the cardiac examination were calculated using Microsoft Excel.

According to the variational pulse oximetry study, the main indicators were determined, such as mode (Mo) - the interval that most often occurs in the R-R interval of the heartbeat, i.e. the duration of the heartbeat recorded by the cardiograph on paper; mode amplitude (AMo) – the percentage value of the mode that develops the mode, i.e. the percentage value of the most frequent value; variation range (Δx) – the difference between the maximum and minimum value of the mode; autonomic balance index (ABI) – an indicator that reflects the influence of the sympathetic and parasympathetic nervous system on the body, determined by the difference between the amplitude of the mode and the variation range; autonomic rhythm index (ARI) – an indicator that reflects the influence of the sympathetic nervous system on the body, determined by formula 1:

$$ARI = 1 \div (\text{Mo} \times \Delta\text{x}); \quad (1)$$

stress index (SI) – an indicator that reflects the state of stress of the body, i.e. reflects the stress index characterising the tone of the autonomic nervous system, is determined by formula 2:

$$SI = \text{AMo} \div (2 \times \text{Mo} \times \Delta\text{x}). \quad (2)$$

Analysing the results by the indicators obtained during the research, research groups were established. The first indicator that helped to determine the tone of the autonomic nervous system was the tension index, which reflected the sympathetic-vagal balance. Animals with low scores belonged to the group of vagotonics, those with high scores belonged to the group of sympathotonics, and cows with intermediate scores belonged to the group of normotonics. Out of 100 cows, 5 animals from each experimental group were selected for a detailed examination of the tone of autonomic nervous regulation. Statistical processing of the experimental data was performed by conventional methods of variation statistics. The significance of the difference was assessed by the Student's t-test. Differences between the compared indicators were considered probable at the level of significance $P<0.05$, $P<0.01$, $P<0.001$.

Results and Discussion

During the research of Ukrainian Black-and-White dairy cows of 3-4 lactation, it was established that the vast majority of animals were characterised by the predominant influence of the sympathetic division of the autonomic nervous system and belonged to the sympathotonic group (Fig. 1). The proportion of cows with sympathotonia was 50%. In contrast, the proportion of cows with normotony and vagotony was 28% and 22%, respectively.

According to the results of the electrocardiographic study, differences in the variational and pulse rate indices in cows depending on the tone of the autonomic nervous system were obtained (Table 1).

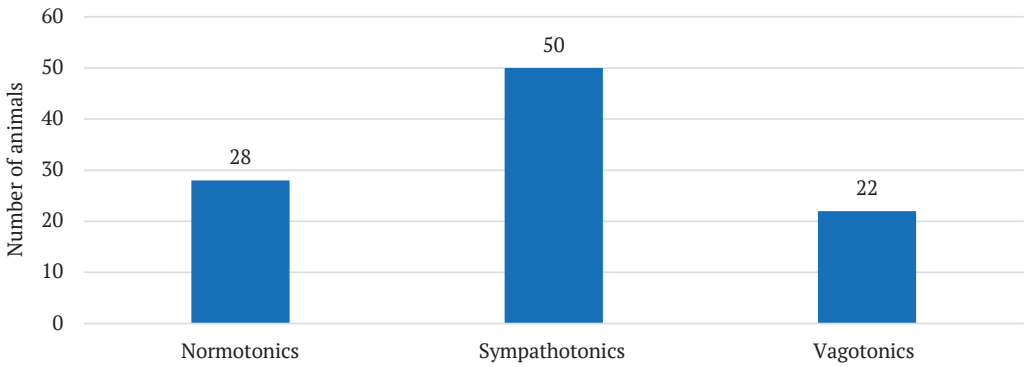

Figure 1. Number of animals depending on the tone of the autonomic nervous system (n=100)

Table 1. Indicators of heart rate variability depending on the tone of the autonomic nervous system in cows (M±m; n=5)

Indicator	Normotonics	Sympathotonics	Vagotonics
Heart rate, beats/min	62.0±3.74	78.0±3.04	50.0±1.60
Mo, c	1.0±0.05	0.78±0.03*	1.20±0.04*
AMo, %	20.40±1.04	32.18±0.89**	11.69±0.38**
Δx, c	0.13±0.01	0.07±0.01*	0.25±0.01**
ABI	153.31±8.06	437.0±22.05	47.93±1.44
ARI	7.95±0.87	18.0±0.89**	3.45±0.15**
SI	79.85±7.90	284.0±19.11	20.0±0.96

***Note**: *P<0.01, **P<0.001 – relative to the normotonic group*
***Source:** author's development*

In sympathotonic animals, the modal parameters of 0.78±0.03 s were the lowest (P<0.01), indicating the predominance of the sympathetic nervous system. The decrease in this value is explained by the fact that the cardiovascular system is dominated by excitation processes. It increases the heart rate. The heart rate increases within physiological standards. In vagotonics, the mode index (1.20±0.04 s) is the lowest compared to other groups (P<0.01) due to the action of the parasympathetic nervous system. Under the influence of a stress factor on the animal's body, the autonomic nervous system is dominated by the phenomenon of vagotonia. The cardiovascular system reduces its excitability, which helps to reduce the heart rate and, accordingly, the pulse rate. There was an inverse correlation between pulse rate and mode, which was r=-0.99 for the experimental groups. Accordingly, the animals of the experimental groups demonstrated a dependence between heart rate and mode index.

The amplitude of the mode characterises the percentage of fashion indicators, reflects the degree of mobilisation influence of the sympathetic nervous system. If sympathotonia prevails in the body, the AMo values will be high. It is explained by the fact that with a high intensity of excitation of the cardiovascular system by the sympathetic nervous system, the interval of repetitions of the cardiac cycle increases. It increases the amplitude of the mode. Accordingly,

high values of indicators were noted in cows of the experimental group of sympathotonics 32.18±0.89% and in small cows of vagotonics 11.69±0.38% (P<0.001). A straight correlation between the mode and the mode amplitude was found in the animals of the experimental groups, namely: normotonics – r=0.92, sympathotonics – r=0.82 and vagotonics – r=0.96.

The variation range describes the difference between the maximum and minimum values of a mode. This indicator describes the influence of the vagus nerve. An increase in this value reflects the predominance of the parasympathetic nervous system over the sympathetic one. Accordingly, the experimental group of vagotonics is defined by vagotonia due to the largest variation range of 0.25±0.01 s (P<0.001).

The autonomic balance index reflects the ratio of the influence of the sympathetic and parasympathetic nervous systems. An animal with sympathotonia has an increase in the amplitude of the mode and a decrease in the variation range. As a result, the value of the autonomous equilibrium index will increase. Therefore, the experimental group of sympathotonics had the highest index (437.0±22.05) due to the superiority of the influence of the sympathetic nervous system over the parasympathetic one. An indirect correlation between the index of autonomous balance and the variation range in animals of the experimental groups was found, namely: normotonics – r=-0.73, sympathotonics – r=-0.80 and vagotonics – r=-0.74. A straight correlation between the autonomic balance index and the mode amplitude was established: in normotonics – r=0.82, sympathotonics – r=0.52, vagotonics – r=0.66.

The autonomic rhythm index reflects the influence of the parasympathetic nervous system on the body. As its activity increases, the indicator decreases. In the experimental group, vagotonics had the lowest value of the autonomous rhythm index – 3.45±0.15 (P<0.001). It indicates the predominance of the parasympathetic nervous system in the animal's body. An indirect correlation of the autonomous rhythm index and the variation range was identified in the animals of the experimental groups, namely: normotonics – r=-0.57, sympathotonics –r=-0.76 and vagotonics – r=-0.77. An indirect correlation between the autonomous rhythm index and mode was determined: in normotonics – r=-0.65, in sympathotonics – r=0.70, in vagotonics – r=-0.58.

The tension index characterises the tone of the autonomic nervous system. Its main value – the determination of the stress index, which indicates the predominance of the sympathetic or parasympathetic nervous system. Animals with high stress index values demonstrate increased aggressiveness and reduced stress resistance, resulting in increased nervous fatigue. Considering this, the experimental group of sympathotonics was distinguished by high values of the tension index (284.0±19.11), which indicates the predominance of the sympathetic nervous system, and vagotonics had the lowest values (20.0±0.96), which indicates the influence of the parasympathetic nervous system.

When considering the variation histogram of sympathicotonic animals (Fig. 2), some features should be noted. First of all, they had small R-R intervals. It, in turn, reflects the increased activity of the cardiovascular system. As the processes of excitation of the sympathetic division of the autonomic nervous system prevailed, the heart rate increased accordingly. Thus, the mode indicators demonstrated slight differences in the size of the interval but varied in the number of intervals. This feature of the variation histogram reflects the predominance of the sympathetic nervous system over the parasympathetic one.

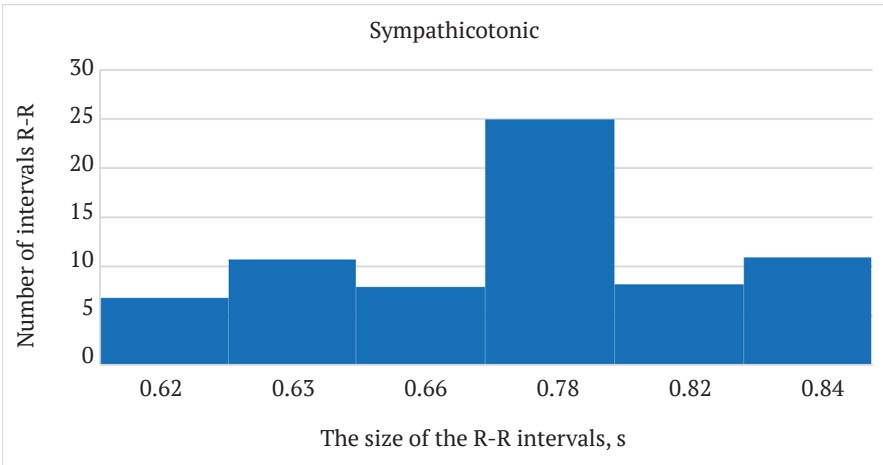

Figure 2. Variational histogram of sympathotonics in the experimental group

The variation histogram of the vagotonics of the experimental group (Fig. 3) demonstrated an increase in the size of the R-R intervals. This feature is explained by a decrease in the activity of the cardiovascular system against the background of vagotonia. The cardiac muscle contracted less actively and at different intervals, which in turn was reflected in the high values of the mode and the value of the R-R interval. It resulted in a decrease in the number of R-R intervals compared to that of animals in other experimental groups. It reflects the predominance of the parasympathetic nervous system over the sympathetic one in the animal's body, as demonstrated by the histogram above.

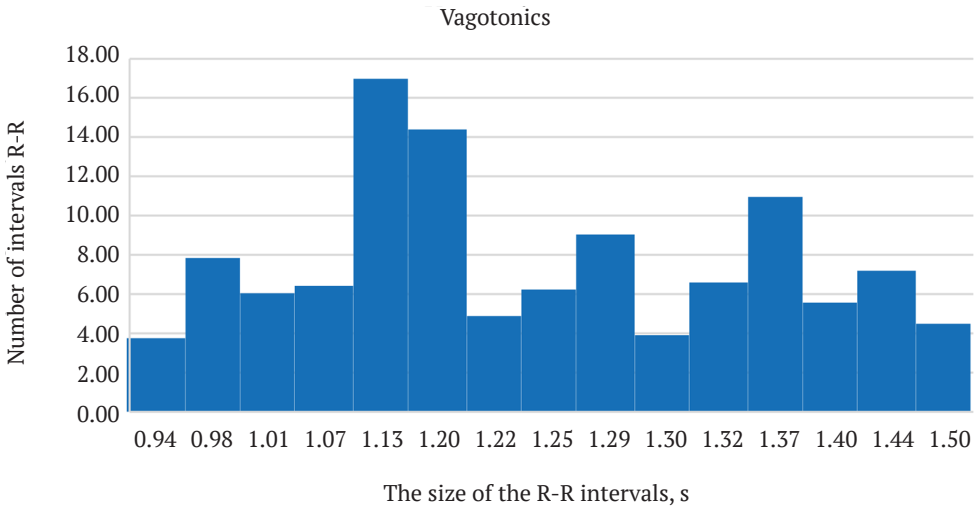

Figure 3. Variational histogram of vagotonics of the experimental group

The experimental group of normotonics on the variation histogram, compared to the others, was distinguished by a uniform ratio of the size of the R-R interval and its number (Fig. 4). This experimental group of animals was characterised by a balanced sympathetic-vagal balance, which in turn affected the cardiovascular system. If the sympathetic and parasympathetic nervous systems have a uniform effect on the cow's body, the heart muscle has a uniform R-R interval frequency. It has a corrective effect on the amount of the R-R interval. Considering the above, normotonics were distinguished by a balanced histogram of heart rate compared to animals of other experimental groups.

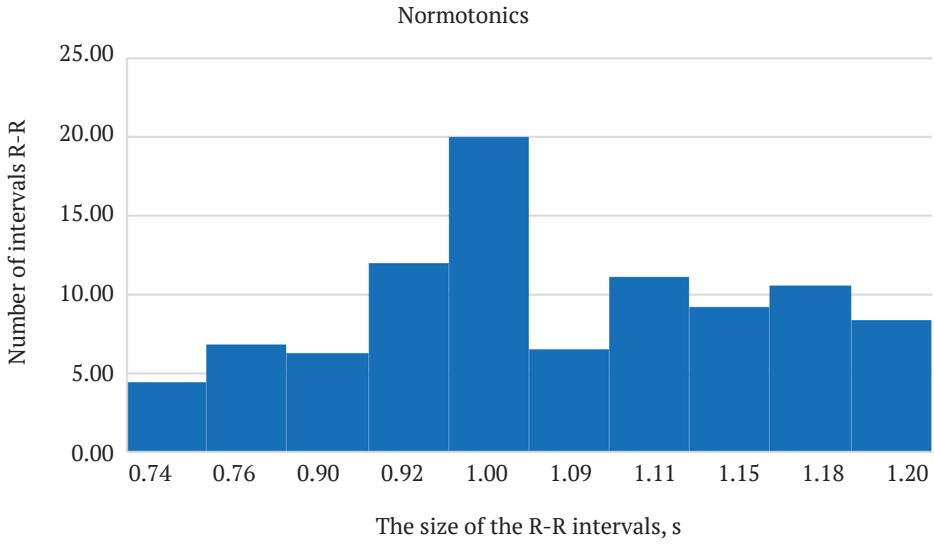

Figure 4. Variation histogram of normotonics of the experimental group

As is known, the issue of assessing the impact of stress on the body of cows is actively developing. The analysis of heart rate during milking or pain factor was frequently used to diagnose the condition of the animal. Over time, the question of better analysis of the cardiovascular system to explore the effects of stress on cows has arisen (Kovács *et al.,* 2019; Scoley *et al.,* 2019). Heart rate variability reflects the activity of the autonomic nervous system in the body. Depending on the influence of the sympathetic or parasympathetic nervous system, an animal responds differently to a stressor. Measurement of these indicators is successfully used to diagnose the health status of an animal and the effectiveness of treatment. It is necessary for a better understanding of the role of the autonomic nervous system in the body, as its influence significantly affects cow productivity (Quevedo *et al.,* 2019; Scoley *et al.,* 2019).

The results obtained in the variational pulse oscillometric test may vary from one researcher to another. It is explained by the fact that depending on the chosen methodology and using electrocardiographs of different models and years, different digital data can be obtained. Despite this, there is a certain regularity in the determination of sympathetic-vagal balance and heart rate. Thus, the experiments of Grelet *et al.*

(2022) noted that animals exposed to stress factors had a disorder of sympathovagal balance, as evidenced by the results of the variational pulse examination. Notably, at week 0, no differences in heart rate were observed between the groups of cows. At the last stage of the research, the animals exposed to the stress factor had a heterogeneous heart rate and a decrease in the values of sympathovagal balance. When comparing the results obtained with the studies of Grelet *et al.* (2022), notably, the animals exposed to stress had low cardiovascular activity. The researchers noted that the changes in the data obtained between the control and experimental groups during the four weeks of stress changed by only 8.2%. It indicates that the distribution of animals without consideration of the tone of the autonomic nervous system does not give a significant result when assessing the effect of a stressor. Therewith, it is important to consider the factor of fluctuations in sympathetic-weight balance, and the data of variability-pulse oximetry, which, according to the results of the research, corresponded to fluctuations between the groups within 21%.

Kovacs *et al.* (2015) used a variational pulse oscillometric examination as an indicator of the effect of chronic stress on the body of cows. In animals with a predominance of the parasympathetic nervous system over the sympathetic nervous system, a decrease in heart rate and high values of sympathovagal balance were observed, which confirmed vagotonia in the experimental groups. The research on the activity of the autonomic nervous system in cows before and during milking identified some differences in the indicators. The researchers note that animals with high activity of the sympathetic nervous system have the highest heart rate and low levels of variation. According to the results of the author's research, the difference in values between the study groups was 21 and 22%, respectively, in contrast to Kovacs *et al.* (2015), who obtained differences in values between the groups of 9 and 14%.

Jurkovich *et al.* (2017) used a variation-pulse study to assess the stress state of cows by changing the type of milking. It appeared that the connection of animals to automated milking machines was manifested by a decrease in heart rate and an increase in sympathetic-vagal balance, unlike those in conventional milking parlours. In cows that had more contact with humans, increased activity of the sympathetic nervous system was observed, which was reflected in aggressive behaviour and an increase in the content of glucocorticoids in faeces. To assess the state of the autonomic nervous system, the cardiovascular system was assessed, and the sympathetic-weight balance was determined. In particular, the authors noted that the cows of the experimental groups demonstrated an increase in the value of the examined indicators during prolonged exposure to a stress factor on the body. Therewith, an increase in pulse rate by 15% and sympathetic-weight balance by 35% was recorded. The experimental group of sympathotonic cows in research was a good reflection of the high activity of the sympathetic nervous system, which allowed obtaining slightly higher values of pulse rate – up to 25% and sympathetic weight balance – up to 38%, compared to other experimental groups of animals.

When analysing the variability of the heart rate of cows, an increase in the value of its indicators was observed in sympathotonics and a decrease in vagotonics. Therewith, normotonics occupied an intermediate position compared to the results typical of animals in other experimental groups. Considering the already-known results of Jurkovich *et al.* (2017) and their comparison with the data of the author's research, similar patterns were noted. When evaluating the results of other researchers, notably, the results of their studies vary due to different methods of determining the tone of autonomic nervous

regulation. When calculating the initial data, most scientists use the software installed in the cardiograph to calculate the parameters of the heart's electrical potentials. Accordingly, the units of measurement will be various, but there is a particular pattern in determining the activity of the autonomic nervous system. The opposite results were observed in vagotonic cows compared to sympathotonic cows. Notably, regardless of the breed of animal and the size of the farm, there is a distribution of animals according to the tone of the autonomic nervous system. In addition, it reflects the individual characteristics of each cow (Kovács *et al.,* 2019). The established patterns, in turn, will serve as a reflection of the animal's health status and the reaction of its body to stress factors that will arise during milking, treatment, regrouping, etc.

After analysing the results, it can be concluded that among the animals of the experimental groups, sympathotonics demonstrated the highest level of nervous tension. It was confirmed by the indicators of the variational pulse study, which provide the initial data describing sympathotonia in such cows. As a result, the cows demonstrated constant nervous tension, which was expressed in a decrease in the heart rate interval and an increase in its frequency. In this regard, the activity of the cardiovascular system increased. During such loads, the animal expended more energy under the influence of external stress factors, which can adversely affect the health of its body and milk production.

Conclusions

According to the results of the studies, it was found that the tone of the autonomic nervous system affects the cardiovascular system, which is confirmed by the results of a variation pulse examination. Therewith, animals with sympathotonia had high values of heart rate – 78.0±3.04 beats/min, R-R interval intensity – 32.18±0.89 s and low values of R-R interval – 0.78±0.03 s ($P<0.01$; $P<0.001$). Animals with vagotonia demonstrated the opposite features, which was characterised by a decrease in heart rate to 50.0±1.60 beats/min, intensity of the R-R interval to 11.69±0.38 s and an increase in the R-R interval to 1.20±0.04 s ($P<0.01$; $P<0.001$). Animals with a balanced influence of the sympathetic and parasympathetic nervous systems occupied an intermediate position in terms of the examined indicators compared to animals of other experimental groups. In addition, it was established that the values of the variational pulse examination are a reliable reflection of the influence of the autonomic nervous system on the entire organism. Depending on the influence of the sympathetic or parasympathetic nervous system, a prediction of the animal's reaction to a stressor is possible. In addition, it allows determining the reaction of the cow's body to changes introduced to its environment, for example, new milking machines, regrouping into new housing units, etc. This method allows for the analysis of changes in animal metabolism under the influence of various exogenous factors and can be used to further explore the effect of new factors in improving the productivity of the dairy cow herd and their welfare.

In the future, as a continuation of experimental research in this area, it is planned to explore the influence of the tone of autonomic nervous regulation, which is determined using a variation-pulse study, on the homeostasis of the body of cows with different indicators of their productivity.

Acknowledgements

None.

Conflict of Interest

None.

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Визначення варіабельності серцевого ритму як показника впливу тонусу автономної нервової системи в корів

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Анотація. Актуальність теми полягає у важливості вивчення ролі автономної нервової системи в регулюванні діяльності серцево-судинної системи для покращення благополуччя продуктивних тварин. Варіабельність серцевого ритму є неінвазійним методом дослідження, що може бути корисним під час дослідження стану здоров'я тварини та аналізу її психофізіологічного стану в умовах господарства. Мета дослідження – встановити вплив тонусу автономної нервової регуляції на організм корови, що відображається в змінах симпато-вагального балансу. Експериментальні дослідження проводили на коровах породи українська чорно-ряба молочна. Для дослідження варіабельності серцевого ритму використовували електрокардіограф із подальшим визначенням основних показників за методикою Баєвського, що включало визначення моди, амплітуди моди, варіаційний розмах, індекс автономної рівноваги, автономний показник ритму та індекс напруги. За

результатами дослідження було сформовано три дослідні групи тварин: нормотоніки, ваготоніки та симпатотоніки. З огляду на отримані результати, корови, залежно від впливу тонусу автономної нервової системи, мають відмінності в діяльності серцево-судинної системи. За рахунок цього буде різнитися їх відповідь на дію фактору стресу, що у свою чергу відображатиметься на їхній продуктивності. Визначення варіабельності серцевого ритму може стати одним з незамінних показників під час аналізу стану здоров'я тварини на молочнотоварних фермах. Це питання є перспективним напрямом досліджень, особливо при вивченні метаболічних процесів організму високопродуктивних корів для покращення ефективності продуктивності зі збереженням фізіологічного стану тварини

Ключові слова: симпато-вагальний баланс; нормотоніки; ваготоніки; симпатотоніки; здоров'я тварин; велика рогата худоба

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Current state of scientific research and prospects for using basidiomycetes in veterinary medicine: A literature review

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Abstract. The relevance of the study is conditioned upon the need to summarise current data on the pharmacological properties of *Basidiomycota* and to provide scientific justification for their use in veterinary medicine. The purpose of the research was to analyse the results of recent experimental studies on the pharmacological and toxicological properties of basidiomycetes and the possibilities of their use in veterinary medicine. The research uses theoretical methods of analysis and comparison of literature data with the development of substantiated conclusions and recommendations for the future. Basidiomycetes belong to the higher fungi, their vegetative body is represented by a branched cellular mycelium. They are a healthy food and rich in biologically active substances. Analysis of the literature demonstrates a high degree of scientific interest in the research of their pharmacological properties. Consequently, scientific research is designed to establish new data on the pharmacodynamics and pharmacokinetics of biologically active substances of basidiomycetes. For example, the mushroom *Inonotus obliquus (I. obliquus)* has been commonly used to treat patients with various gastrointestinal diseases. In recent years, there has been a growing body of scientific evidence indicating the effectiveness of its extracts in treating

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infections and infestations, cancer, and diabetes. It was established that the biologically active substances of *I. obiquus* stimulate the immune system, show hypoglycaemic potential, are sensitive to insulin, and have an antioxidant effect. In the current scientific discourse, researchers are trying to identify the molecular mechanism of action of *I. obliquus* extracts. The presented review of the literature demonstrates that basidiomycetes exhibit pharmacological properties in cancer and neurodegenerative diseases, diseases of the digestive, respiratory and cardiovascular systems, metabolic disorders, antimicrobial, antiviral, immunomodulatory effects, etc. Currently, using basidiomycetes in the world, considering their scientifically proven and experimentally proven pharmacological properties, is extremely promising. The material of the research is of practical value for expanding the possibilities of using fungotherapy in veterinary medicine

Keywords: fungi; prevention; diseases; fungotherapy; biologically active substances; molecular mechanisms

Introduction

With the continuous development of veterinary medicine, new ways of preventing diseases and treating sick animals are emerging. Along with the expansion of the pharmaceutical market for synthetic medicines, the number of new veterinary medicines of natural origin is growing. Since the first use of penicillin in medical practice, which was obtained from the mouldy fungi of the genus *Penicillium*, fungus-based medicines have remained relevant. The results of modern scientific research indicate their new pharmacological properties (Alvandi *et al.,* 2020; Lu *et al.,* 2021). It is a prerequisite for expanding the possibilities of their use in modern veterinary medicine.

A distinct class of fungi that have not previously received sufficient attention from scientists, physicians and veterinarians is the *Basidiomycota*. They belong to the division of higher fungi and have a vegetative body represented by a branched cellular mycelium. The evolutionary development of basidiomycetes occurred in parallel with the evolution of ascomycetes, or marsupial fungi. Nowadays, this division of fungi is the second largest after the Ascomycota in terms of the number of species; it includes about thirty thousand species belonging to almost all ecological groups of fungi, including saprotrophs on various substrates, parasites of higher plants, mycorrhizal mycorrhizae, wood-destroying fungi, coprotrophs, mycotrophs, etc. It is well known that many species of basidiomycetes are poisonous or edible fungi, but some of them have pharmacological properties (Doğan *et al.,* 2020; Lu *et al.,* 2021).

Currently, chemotherapy, which has many disadvantages, is being replaced by new methods that have a direct effect on tumour cells (Xiong *et al.,* 2016; Bian *et al.,* 2022; Chis *et al.,* 2022). Therefore, therapies based on biopolymer systems are an alternative. In addition, current trends are focused on natural sources, which are considered biocompatible, biodegradable, valuable and renewable sources (Alvandi *et al.,* 2020; Zhao & Zheng, 2021). Chis *et al.* (2022) analyse recent studies focusing on polymer-drug conjugates containing natural polymers such as chitosan, hyaluronic acid, dextran, pullulan, silk fibroin, heparin and polysaccharides from *Auricularia auricula (A. auricula)*.

In recent years, a large number of studies have been conducted to establish their pharmacological effects as antibiotics, antiviral or antitumour agents, etc. (Fig. 1)

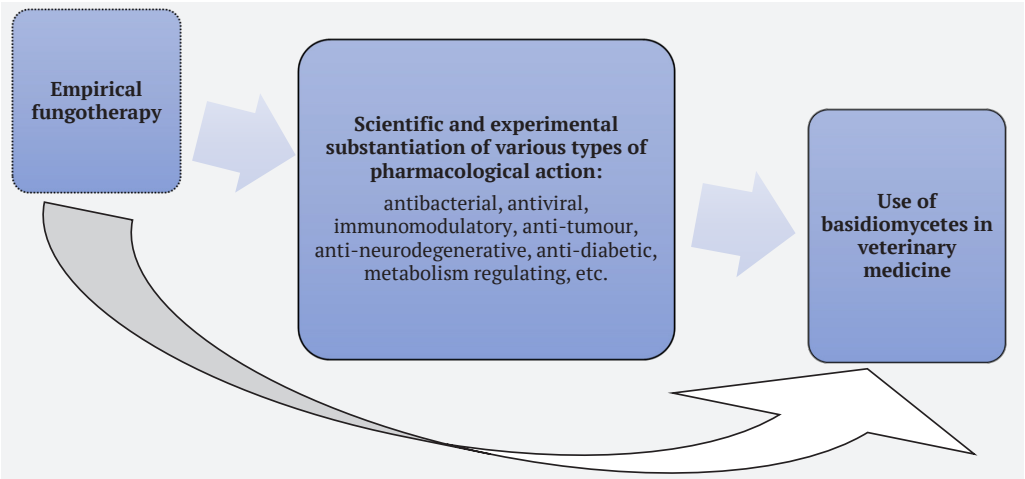

Figure 1. Prerequisites for using basidiomycetes in modern veterinary medicine
Source: *compiled by the authors based on the analysis of literature*

The purpose of the research was to analyse the literature data on the results of modern scientific studies of the pharmacological properties of basidiomycetes and promising areas of their use in veterinary medicine.

Efficiency of basidiomycetes in cancer treatment

Historical chronicles describe the treatment of Prince Volodymyr Monomakh of Kyiv with the mushroom *Inonotus obliquus* (*I. obliquus*) for a tumour on his lip. It has now been established that chemically diverse bioactive metabolites provide *I. obliquus* with great potential for inhibiting tumour growth and metastasis. Thus, Zhao & Zheng (2021) proved that *I. obliquus* has the potential to reduce the incidence of tumour development in healthy people. For people whose complete remission has been achieved by chemotherapy, the administration of the fungus inhibits the activation of advanced oncogenic signals, preventing metastases. After chemotherapy, fungus supplementation sensitises tumour cells, enhancing chemotherapeutic effects and helping to restore weakened immunity and protect against gastrointestinal ulcers and other side effects caused by chemotherapy.

A. auricula – a fungus of the Auriculariaceae family, which is common in Eurasia. It contains biologically active substances such as polysaccharides, melanin, flavonoids, adenosine, sterols, alkaloids, and terpenes. Liu *et al*. (2021) explored the properties of lectins (AAL) isolated by affinity chromatography from *A. auricula*. Their protein identification results, according to the UniProt database, demonstrated that the lectin contains four peptide chains, and the analysis of its physical and chemical properties showed that AAL is sensitive to changes in temperature and pH. The antitumour activity assay demonstrated that AAL inhibited the proliferation of A549, with an IC50 value of 28.19±1.92 μg/mL. Due to RNA sequencing and analysis of the TCGA dataset, it was identified that AALs can regulate the expression of JUN, TLR4 and MYD88 to suppress tumour proliferation. Thus, the lectin protein isolated from the basidiomycete *A. auricula-judae* regulates the expression of some cancer-related genes and has anti-tumour properties. Therewith, researchers predict its successful use to influence the lung microflora.

A conjugated complex of folic acid (FA) with the Auricularia auricular polysaccharide (AAP) – cis-diamidine dichloroplatin (CDDP) (FA-AAP-CDDP) was used for the treatment of cervical carcinoma (Qiu *et al.*, 2018). With this combination, the researchers could increase the anti-tumour effect of CDDP and reduce the toxic side effects of CDDP. The kidneys of mice treated with the FA-AAP-CDDP complex had increased superoxide dismutase, catalase and glutathione peroxidase activity and lower levels of malondialdehyde. The FA-AAP-CDDP complex has the ability to induce more interleukin-2, interleukin-4 and interferon-γ in mice. In addition, the FA-AAP-CDDP complex significantly promoted the expression of Bax and caspase-3 proteins but inhibited the expression of Bcl-2 protein, which activated the mitochondrial apoptotic pathway of tumour cells in laboratory mice. These results provide a new area for the research of polymers targeting folic acid receptors to increase the antitumour activity but reduce the side effects of CDDP.

Qiu *et al.* (2018) explored the anti-tumour activity of *Calvatia gigantea (C. gigantea)* extract, which belongs to the Lycoperdaceae family. Changes in CCND1, CCND2, CDK4, p21, Akt, Bax, Bcl-2, p53, caspase-3 and caspase-9 were assessed in A549 lung cancer cells using CTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide). The results obtained on A549 cells treated with *C. gigantea* extract at a dose of 500 μg/mL for 72 h presented that the expression of CCND1, CCND2, CDK4, Akt and Bcl-2 decreased, while the expression of Bax, p53, caspase-3 and caspase-9 increased. However, no changes in p21 expression were observed. The examined *C. gigantea* extract induced cell cycle arrest and apoptosis by decreasing the expression of CCND1, CCND2, CDK4, Akt and Bcl-2 and increasing the expression of Bax, p53, caspase-3 and caspase-9 in A549 cells. Eroğlu *et al.* (2016) believe that *C. gigantea* extract can be an effective treatment for lung cancer patients and can be used both alone and in combination with other drugs.

It is well known that the edible tinder fungus *Fomes fomentarius* (*F. fomentarius*) has anti-cancer, anti-inflammatory and anti-diabetic effects. Lee *et al.* (2019) explored its effectiveness in cancer treatment. To determine the molecular mechanism of the anticancer effects of *F. fomentarius*, the researchers used various methods, including activated fluorescence cell sorting, Western blot, migration and crystal violet analysis. Ethanolic extract of *F. fomentarius* ethanolic extract (FFE) decreased cell viability in six cancer cell lines (MDA-MB-231, MCF-7, A549, H460, DU145 and PC-3). FFE reduced the migration of MDA-MB-231 cells without causing cellular toxicity. In addition, FFE attenuated the expression of matrix metalloproteinase-9 and Akt phosphorylation and enhanced E-cadherin in MDA-MB-231 cells. FFE arrested S and G2/M populations by inhibiting the expression of cell cycle regulatory proteins such as cyclin-dependent kinase 2, cyclin A/E and S-phase kinase-associated protein 2. FFE increased the sub-G1 population and the expression of cleaved caspase-9, -3 and cleaved polyadenosine diphosphate (ADP-ribose) polymerase after 72 h and suppressed B-cell lymphoma 2. FFE and AKT inhibitors caused similar effects in MDA-MB-231 cells. In addition, FFE contained betulin, which inhibited p-AKT in MDA-MB-231 cells. The results obtained by the authors showed that FFE inhibits cell motility and growth and induces apoptosis by inhibiting the phosphoinositide 3-kinase/AKT pathway and activating caspase.

Alvandi *et al.* (2020) established that the antiproliferative effect of fungal polysaccharides on cancer cells depends on the type of cancer and increases with increasing concentration. The results of initial experiments on biomass and polysaccharide production by *F. fomentarius* demonstrated that after 4 days

of cultivation in the desired culture medium, biomass production reached 15 g/L, and polysaccharide production reached 4.06 g/L. Thus, a culture medium including glucose, peptone, malt extract, yeast extract, MgSO4-7H2O and KH2PO4 is a suitable culture medium for the growth and production of polysaccharides by this fungus. Supplements made from tinder fungus polysaccharides with selenium can be used to inhibit the proliferation of cancer cells.

Rehman *et al.* (2020) proposed an alternative method for the synthesis of nanoparticles (NPs). In particular, titanium oxide nanoparticles and silver nanoparticles (TiO_2 and Ag nanoparticles, respectively) were obtained by mixing the intracellular extract of a wild fungus, *F. fomentarius* – with aqueous solutions of titanium isopropoxide and silver nitrate, respectively. Success was achieved in obtaining NFs of various shapes and forms and their antibacterial and antitumour activity was established. The researchers argued that NFs synthesised using wild fungi have great potential in biomedicine due to the effective combination of enzymes that allows them to modify various chemical compounds into less toxic forms, which is essential for environmentally friendly and safe biomaterials for drug synthesis. To evaluate the antitumour activity and study drug resistance, Doğan *et al.* (2020) prepared aqueous, methanolic and ethanolic extracts of *F. fomentarius* and *Tricholoma anatolicum (T. anatolicum)*. It was established that the methanolic extract of *F. fomentarius* and ethanolic extract of *T. anatolicum* have potential value in the development of drugs for the treatment of breast cancer patients and can be used as a natural multidrug resistance modulator (MDR modulator).

The Chaga mushroom – *Inonotus obliquus* (*I. obliquus)* – is known as a foodstuff and is used for medicinal purposes, especially in China, Korea and some Western countries. Among the *I. obliquus* extracts, *Inonotus obliquus* polysaccharide (IOPS) is considered to be one of the main bioactive components of *I. obliquus*, which has antitumour, antioxidant, antiviral, hypoglycaemic and hypolipidaemic effects. The review by Lu *et al*. (2021) summarised the current advances in the extraction, purification, structural characteristics and biological activity of IOPS, which provides significant insight into the biological activity of IOPS as it summarised *in vitro* and *in vivo* data and listed some possible mechanisms. In addition, the potential applications of IOPS were analysed and it was found that IOPS could be a potential drug for the treatment of cancer and type 2 diabetes.

Inonotus obliquus is a parasitic fungus that grows on birch trees and is used in traditional medicine (especially by the Khanty) for therapeutic purposes for various pathologies. The purpose of the research conducted by Géry *et al.* (2018) was to quantify 3 metabolites often described in the literature, namely betulin, betulinic acid and inotodiol in chaga found in the forests of Normandy (France) and compare their concentrations with Ukrainian and Canadian Chaga. The cytotoxicity of French chaga against cancer cells and transformed cells was explored. It was established that French chaga contains more betulin and betulinic acid than chaga from other localities, while the concentration of inotodiol was higher in Canadian Chaga. In addition, the results highlighted the cytotoxic activity of the aqueous Chaga extract after 48 and 72 h of exposure, with a higher effect on A549 cancer cells than on normal transformed BEAS-2B cells (P=0.025 after 48 h of exposure and P=0.004 after 72 h of exposure).

Efficiency of basidiomycetes for metabolic disorders

Obesity

Obese animals develop metabolic changes that can cause significant adverse effects at various levels in their bodies.

Researchers Liu *et al.* (2022) explored the effect of polysaccharides derived from the mushroom Auricularia auricula-judae on dietary obesity in C57BL/6J mice induced by high-fat, high-fructose diets (HFFD). The results demonstrated that dietary supplementation with Auricularia auricula-judae polysaccharides (AAP) significantly improved the insulin resistance of mice, altered serum lipid metabolites and delayed the weight gain induced by HFFD. In addition, AAP supplementation reduced inflammatory factor levels and alleviated changes in liver histomorphology. Therewith, AAP suppressed the expression of genes related to liver adipogen, inhibited the levels of genes responsible for cholesterol synthesis, enhanced the expression of genes related to fatty acid β-oxidation, and promoted the expression of genes affecting cholesterol efflux, which improved the homeostasis of lipid metabolism in the liver of mice. Liu *et al.* (2022) emphasise the importance of mitochondrial functions in this process, and the results obtained by the researchers provide a scientific basis for the further development and utilisation of *Auricularia auricula-judae* resources.

Yu *et al.* (2022) explored the effects of two extracts of *Inonotus obliquus* (*I. obliquus,* chaga, birch black mushroom) on mice induced by a high-fat diet. They were administered an ethanolic extract (IOE) and a polysaccharide (IOP) of *I. obliquus*. The authors established that IOE and IOP effectively corrected the increase in body weight and energy use in the body, affected the metabolism of glucose in the liver and triglyceride metabolism, the tricarboxylic acid cycle, and the metabolism of carbohydrates, lipids and proteins in general. Two extracts containing different bioactive substances of *I. obliquus* reduced obesity in mice due to different effects on the synthesis of microbial metabolites in the intestine.

Diabetes

Type 2 diabetes mellitus is an urgent problem, therefore, scientists are actively developing non-toxic ways to treat animals and humans with this disease. Thus, it has been established that *Auricularia auricula* polysaccharides (AAP) promote recovery from this pathology (Liu *et al.*, 2022).

Liu *et al*. (2022) demonstrated that AAP achieves remission by altering the gut microbiota in mice with type 2 diabetes. As a result, a model of type 2 diabetes mellitus (T2DM) induced by a high-fat diet (HFD) combined with streptozotocin (STZ) was established, and a decrease in fasting blood glucose (FPG) and oral glucose tolerance test (OGTT) was observed after 5 weeks of AAP administration. In addition, AAP enhanced the activity of total superoxide dismutase (T-SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) and reduced malondialdehyde (MDA), which alleviated oxidative stress. The best effect was observed with AAP-M (200 mg/kg/day). Therewith, 16S rRNA results demonstrated that AAP decreased the number of *Firmicutes* and increased the number of *Bacteroidetes*. AAP can reduce the number of *Desulfovibrio*, *Enterorhabdus* and *Helicobacter*. Overall, these results suggest that AAP may improve glucose and lipid metabolic disorders by regulating the gut microbiota structure.

The research by Gery *et al.* (2018) confirms a decrease in fasting glucose and insulin levels and an improvement in insulin resistance in mice.

The identification and authentication of the giant porchavaca fungus using molecular tools was performed and its antidiabetic properties were explored. In an acute *in vivo* test, a dose of 400 mg/kg body weight presented the best activity with a 29.3% reduction in blood glucose levels, while glibenclamide at a dose of 5 mg/kg body weight reduced glucose levels by 15%. The in vitro assay demonstrated that the extract was highly active with an IC50 of 0.46 µg/mL

compared to its DCM, butanol and acarbose fractions (IC50 5.3 μg/mL, 5.6 μg/mL, 45 μg/mL), respectively. Therewith, BLAST analysis demonstrated that the fungus was 98% identical to *Calvatia gigantea*. Thus, Ogbole *et al.* (2019) confirmed its pronounced anti-diabetic effect.

According to Lu *et al.* (2021), a polysaccharide from *Inonotus obliquus* reduces the risk of developing type 2 diabetes by affecting intestinal barrier dysfunction.

Szychowski *et al.* (2021) established two mechanisms of action of *I. obliquus* extracts, which are explained by the effect on antioxidant enzymes and reactive oxygen species levels and by the effects of the peroxisome proliferator-activated receptor gamma (PPARγ). This receptor may be a key factor in the anti-inflammatory, antioxidant and anti-cancer activity of *I. obliquus* extracts. Researchers claim that *I. obliquus* has potentially positive health effects, but there is a need for studies that meet the criteria of evidence-based medicine.

The protective effect of chaga on the kidneys has been confirmed by biochemical data, colour Doppler ultrasound, light and electron microscopy, and histopathological studies (Su *et al.,* 2022). In addition, this effect of Chaga is associated with its effect on slowing the progression of kidney damage. An 8-week administration of chaga demonstrated excellent hypoglycaemic and hypolipidaemic effects in rats with type 2 diabetes mellitus induced by a high-fat diet (Su *et al.*, 2022; Zhang *et al.*, 2022).

The study conducted by Su *et al.* (2022) isolated a single homogeneous polysaccharide from Inonotus obliquus (IN) and explored the protective effect and mechanism of IN on type 2 diabetes mellitus from the intestinal barrier. IN, which was mainly composed of 9 monosaccharides with an Mw of 373 kDa, reduced body weight loss, alleviated pathological damage and suppressed the production of pro-inflammatory cytokines. In addition, IN contributed to the restoration of the intestinal barrier by increasing the expression of Ki-67, ZO-1 and MUC2. The number of *Firmicutes* increased significantly during treatment with IN, while the levels of *Bacteroidetes* were significantly suppressed. The scientists concluded that IN protects against type 2 diabetes by reducing the degree of intestinal barrier dysfunction and can be recommended as a new drug for the treatment of animals and humans with type 2 diabetes.

Diabetic kidney disease (DKD) is currently the leading cause of end-stage renal disease. To explore the therapeutic potential of *Inonotus obliquus* (Chaga) in the treatment of diabetic patients, Zhang *et al.* (2022) used Chaga extract to determine the renal protective effects in a rat model of DKD induced by a high-fat diet and streptozotocin injection. For the 17-week experiment, biological parameters of blood serum and urine were examined, renal arteries were colour Doppler, periodic acid-Schiff staining, and electron microscopy of kidney tissue were performed. The results of biochemical studies demonstrated that, compared to the control group, the DKD model developed insulin resistance, increased levels of glucose and total cholesterol in the blood, and increased urinary protein excretion and serum creatinine levels. Ultrasound examination confirmed the deterioration of blood flow parameters of the left intercalated artery in rat models. It has been established that ultrasound Doppler examination using biochemical parameters has an important role in the assessment of renal damage in rats. Based on the biochemical, ultrasound and histopathological data, the therapeutic effect of Chaga on diabetes-induced renal damage was confirmed, and the above effects may be associated with delaying the progression of DKD.

Antioxidant features

Su & Li (2020) explored the structural characterisation and antioxidant activity of four

Auricularia polysaccharides (*A. cornea*, ACP; *A. auricula*, AAP; *A. polytricha*, APP; M. *fungus*, MFP). The results obtained by the researchers demonstrated that *Auricularia* polysaccharides consisted mainly of mannose and galactose, containing uronic acid and a pyran ring structure. A significant difference was observed in the total antioxidant capacity, in particular, in ARP it was significantly higher than in other polysaccharides. It was found that the ability of ARR to scavenge DPPH and hydroxyl radicals was higher than that of other polysaccharides, respectively. Molecular weight was significantly positively correlated with DPPH radicals, superoxide anion radicals and hydroxyl radicals. Total antioxidant capacity was significantly negatively correlated with fucose and galactose. The results demonstrated that fucose and galactose jointly determine the total antioxidant capacity. The polysaccharide from four auriculars presented resistance to oxidation and is recommended by the authors as a natural antioxidant.

Extracellular polysaccharides of *Fomes fomentarius* have a rather pronounced antioxidant activity (Alvandi *et al.,* 2021). Zhang *et al.* (2020) explored the isolation, cultivation, and purification of extracellular polysaccharides from *Fomes fomentarius*. They optimised the extraction of polysaccharides from the culture based on extracellular polysaccharides of *F. fomentarius*, comparative experiments on the fermentation of extracellular polysaccharides were conducted and the optimal fermentation method was obtained; extracellular polysaccharides were sulfated, experiments with phosphorylation, selenium acidification were conducted, the preparation of polysaccharide derivatives was examined, etc. The results obtained by the authors presented that the extracellular polysaccharide and derivatised polysaccharide of *F. fomentarius* have certain antioxidant activity.

Efficiency in diseases of the stomach and intestines

Using the polysaccharide of *A. auricula-judae* to maintain and regulate the composition of the intestinal microbiota is promising. In particular, Liu *et al.* (2022a) found the ability to regulate the structure and composition of the gut microbiota in diabetic mice and increase the number of beneficial bacteria that produce short-chain fatty acids, including Lachnospiraceae, Faecalibaculum, Dubosiella and Alloprevotella, which play a crucial role in maintaining the composition of the gut microbiota.

Liu *et al.* (2022b) explored the effects of polysaccharides extracted from *A. auricula* on the overall metabolism and gut microbiota in healthy C57BL/6J mice. Based on the metabolic results of LC-MS/MS, 51 AAP-regulated metabolites were identified, mainly enriched in the arginine biosynthesis pathway, which had the highest correlation, and the following metabolites: arginine and proline; glycine, serine and threonine; glycerophospholipid, etc. Furthermore, the addition of AAP significantly changed the composition of the gut microbiota in mice. The relative numbers of *Lactobacillus johnsonii, Weissella cibaria, Kosakonia cowanii, Enterococcus faecalis, Bifidobacterium animalis* and *Bacteroides uniformis* increased markedly, while the relative number of Firmicutes M10-2 bacteria decreased. The biological activity of AAP may be related to the regulatory effects of endogenous metabolism and gut microbiota composition.

Oral administration of a polysaccharide from *Hericium erinaceus* can affect the diversity and abundance of the gut microbiota. Changes in the flora lead to changes in the proportion of probiotics and modulation of immune responses Yang *et al*. (2021).

Hericium erinaceus polysaccharides (HEPs) were identified by Yang *et al*. (2021) from the fruiting bodies of *H. erinaceus* (hedgehog

geranium, lion's mane). To explore the effect of HEP on the diversity and abundance of the intestinal microbiota, adult, middle-aged, and elderly mice were fed HEP for 28 days. Based on the results of 16S sequencing of the intestinal microbiota, it was determined that the relative abundance of *Lachnospiraceae* and *Akkermansiaceae* increased significantly, while the relative abundance of *Rikenellaceae* and *Bacteroidaceae* decreased. Bacterial flushes from different segments of the mouse intestine and faeces were collected for *in vitro* fermentation of HEP. It was determined that HEP significantly promoted the production of NO, IL-6, IL-10, INF-γ and TNF-α. Moreover, HEP significantly enhanced the phosphorylation of signalling molecules, indicating that the immunomodulatory activity was completed through the NF-kB, MAPK and PI3K/Akt pathways. The researchers note that HEPs can be used as active ingredients in medicines.

Efficiency in diseases of the cardiovascular system

With the increasing number of cardiovascular diseases, the demand for safe and effective drugs for the treatment and prevention of thrombosis has grown. In a study conducted by Bian *et al.* (2022), the structural characteristics and thrombolytic (fibrinolytic) activity of a new polysaccharide isolated from the fruiting bodies of *A. auricula* were determined. The purified polysaccharide AAP-b2 (12.02 kDa) consisted of mannose, glucuronic acid, glucose and xylose with a molar ratio of 89.25::30.50:4.25:1.00. The thrombolytic (fibrinolytic) activity of AAP-b2 has been proven to be associated with inhibition of platelet activation by regulating endothelial nitric oxide synthases (eNO), endothelin-1 (ET-1), prostacyclin (PGI2) and thromboxane B2 (TXB2), along with enhanced anticoagulant activity by affecting antithrombin III (AT-III) and protein C (PC).

Wang *et al.* (2019) explored the effect of *Coriolus versicolor* (CV) on cardiac function in rats with diabetes mellitus (DM). It was established that the heart function in rats with diabetes mellitus was significantly improved by treatment with CV extract. The administration of CV extract significantly attenuated cardiac fibrosis in diabetic rats, which was accompanied by inhibition of transforming growth factor-beta 1 (TGF-β1)/Smad. It was evidenced by a decrease in the levels of TGF-β1, p-Smad2 and p-Smad3 and an increase in Smad7. In addition, an anti-inflammatory effect was observed after treatment with CV extract. In addition, it is known that cardiac fibrosis develops with high glucose content, but the development of this pathology was inhibited by using CV extract. Thus, the pharmacological effect of CV is associated with the inhibition of TGF-β1/Smad signalling and attenuation of NLRP3 inflammatory activation, which suggests that CV extract may be a potential therapeutic agent for cardiovascular diseases.

Efficiency in neurodegenerative diseases

Abitbol *et al.* (2022) used mushrooms in neurodegenerative diseases. In their studies, mushrooms were identified as a rich and largely untapped source of bioactive compounds in both *in vitro* and *in vivo* models of neurodegenerative diseases, which was partially confirmed by successful clinical trial evaluations. Researchers indicate that mushrooms are effective in the treatment of patients with neurodegenerative diseases.

The results obtained by Bai *et al.* (2019) demonstrated that protea-β-glucan, a polysaccharide derived from *Grifola frondosa* (PGM), can improve learning and reduce memory decline, and in laboratory animals, attenuate neuronal loss and histopathological abnormalities in APP/PS1 mice. In addition, PGM treatment can activate microglia and astrocytes and

promote the interaction of microglia and Aβ plaques. It was established that PGM can enhance Aβ phagocytosis and thus reduce Aβ burden and pathological changes in the cerebral cortex and hippocampus in APP/PS1 mice. PGM had no significant effect on the body weight of mice. In summary, these results demonstrated that PGM administration can reduce memory impairment through its immunomodulatory effect, and dietary supplementation with PGM will have a positive therapeutic effect on memory dysfunction associated with brain ageing.

Earlier, Wei *et al.* (2018) reported ten new polyoxygenated cyanoditerpenoids, neocytins A-J, and their anti-inflammatory effects from the liquid culture of the basidiomycete *Cyathus africanus*. Subsequently, the scientists examined 8 new highly polyoxygenated cyanotannoditerpenoids, named neocyanins K-R, which were isolated from a solid culture of *C. africanus* grown on cooked rice, along with three known congeners. All compounds were evaluated for their neurotrophic and anti-inflammatory activities. The isolates obtained at 1-25 μM caused differential nerve growth factor-induced activity. The researchers claim that these substances can be used to treat patients with neurodegenerative diseases.

Li *et al.* (2020) determined the efficacy and safety of a capsule formulation containing *H. erinaceus mycelia* (EAHE) (350 mg/capsule; 5 mg/g active ingredient erynacin A) in the treatment of patients with mild Alzheimer's disease (AD). Trinacin A enriched with *H. erinaceus* is a safe and well-tolerated supplement that may contribute to neurocognitive benefits. Studies have demonstrated higher scores on cognitive screening, mini-mental state examination and activities of daily living, and higher contrast sensitivity in patients with mild Alzheimer's disease compared to patients in the placebo group.

The research by Cordaro *et al.* (2021) showed the effect of the antioxidant mushroom *Hericium erinaceus* on a laboratory rat model of Alzheimer's disease (AD). The animals were administered 70 mg/kg of $AlCl_3$ daily for 6 weeks, and *Hericium erinaceus* was administered daily through a gavage. A behavioural test was performed before the end of the experiment, behavioural changes were assessed at the end of the experiment and the animals were euthanised. Brain tissue was collected for further analysis. $AlCl_3$ mainly accumulates in the hippocampus, the main brain region involved in memory and learning functions. Using *Hericium erinaceus* reduced behavioural changes and neuronal degeneration in the hippocampus. In addition, it reduced the level of phosphorylated tau protein, aberrant overexpression of ARP and β-amyloid accumulation. *Hericium erinaceus* reduced AD-induced pro-oxidative and pro-inflammatory changes in the hippocampus and decreased the activation of NLRP3 inflammatory components, which are usually activated by increased oxidative stress in AD. Overall, the results obtained by the researchers demonstrated that *Hericium erinaceus* has a protective effect on behavioural changes and histological modifications associated with AD through modulation of oxidative and inflammatory pathways, and regulates brain cellular stress.

The mushrooms *H. erinaceus* and *C. versicolor* and their combination can influence specific molecular mechanisms that link chronic traumatic brain injury and Parkinson's disease. In particular, they act on microglia, reducing inflammation that spreads from the cortex to other parts of the brain. In addition, these compounds act on oxidative stress spreading to the midbrain, thus limiting the development of Parkinson's disease. Research conducted by D'Amico *et al.* (2021) suggests that *Hericium erinaceus* and *Coriolus versicolor* may be important in the mechanisms of redox stress response and neuroprotection, preventing the progression of neurodegenerative diseases.

Li *et al.* (2020) explored the effect of Coriolus versicolor plant polysaccharides (CVP) on neuronal apoptosis in a rat model of cerebral ischaemia-reperfusion injury (CIRI) and examined the mechanisms and efficacy of CVP in the treatment of rats with neuronal apoptosis in CIRI, including infarction, inflammation, etc. It was established that CVP is effective already at 6, 12, 24 and 48 hours, which is manifested by an improvement in the behavioural test, a decrease in the volume of cerebral infarction and inhibition of nerve cell apoptosis in CIRI rats. The expression levels of p-p38MAPK protein and cleaved caspase-3 demonstrated a downward trend after CVP administration. CVP significantly reduced the pathological characteristics of CIRI in rats and inhibited apoptosis of nerve cells around the lesions. The mechanism of its effectiveness is associated with the inhibition of activation of the p38MAPK signalling pathway.

Antimicrobial and antiviral efficiency

There are many reports in the literature on the antimicrobial and antiviral efficiency of basidiomycetes. This issue has been particularly explored by researchers.

Thus, the intracellular extract of the wild mushroom *Fomes fomentarius* mixed with aqueous solutions of titanium isopropoxide and silver nitrate has antibacterial effects (Rehman *et al.*, 2020). Tinder fungus polysaccharide inhibits the growth of *S. aureus* and *E. coli* by 50% and 25%, respectively (Alvandi *et al.*, 2020).

Alresly (2019) conducted a study of the biological and chemical potential of two European fungal species: Fomitopsis betulina and Calvatia gigantea. For this purpose, different extracts of both mushrooms were tested for antimicrobial, antifungal, cytotoxic, wound healing and anti-adhesive properties in vitro. Fractionation under bioanalysis control led to the isolation of bioactive compounds, and a total of 20 compounds were isolated and identified. The compounds were obtained from ethyl acetate extracts and included triterpenes, sterols and aromatic compounds. The biological activity of the isolated substances from both fungi was proven, and some of them showed antimicrobial and cytotoxic activity.

The extract from *Inonotus obliquus* has been established to have high antiviral activity, including against COVID-19 (Shahzad *et al.*, 2020; Su *et al.*, 2022).

There is currently no specific treatment for SARS-CoV-2 infection; however, some drugs have demonstrated clear efficacy in suppressing the viral disease. Natural sources of BARs, such as herbs and fungi, have previously demonstrated pronounced antiviral and anti-inflammatory effects. Shahzad *et al*. (2020) assessed the potential for using natural substances as effective treatments for COVID-19. They suggested that one potential source for SARS-CoV-2 drugs could be Inonotus obliquus (IO), a well-known chaga mushroom commonly found in Asia, Europe and North America and widely used as a raw material for the preparation of dosage forms for various diseases worldwide. Considering this, researchers have been evaluating the most effective herbs and mushrooms with antiviral and anti-inflammatory effects in the laboratory.

Impact on the immune system

Traditional medicine has long been interested in the effect of *Auricularia auricula-judae* on the body's immune system. However, in scientific circles, this issue is understudied.

Ibe *et al.* (2020) evaluated the immunomodulatory effects of the hot water extract (AAAJ) and the β-D-glucan-rich polysaccharide fraction of *A. auricula-judae* (BGPA) on specific and nonspecific humoral and cell-mediated immune responses in immunocompetent and immunosuppressed mice. Oral administration of AAAJ or BGPA (100, 200 or 400 mg/kg) induced

significantly higher titres of total OVA-specific or TT-specific IgG1 and IgG2a compared to their levels in untreated controls. Oral administration of AAAJ or BGPA (100, 200 or 400 mg/kg) caused a significant increase in carbon clearance at all doses, indicating stimulation of the reticuloendothelial system, and enhanced the delayed-type hypersensitivity reaction induced by sheep red blood cells (SRBC) compared to untreated mice. Total lymphocyte and neutrophil counts significantly increased ($P<0.05$) at all doses after administration of AAAJ or BGPA (100, 200 or 400 mg/kg), demonstrating increased protection against cyclophosphamide-induced myelosuppression compared to control (untreated). In the haemolytic complement assay, AAAJ and BGPA at all doses significantly ($P<0.05$) inhibited the haemolytic activity of complement proteins on sensitised SRBC. The results obtained by the researchers demonstrated that the extract is a promising immunomodulatory agent for use in veterinary medicine.

Thus, supplements with *Fomes fomentarius* polysaccharides with added selenium have been established to enhance immune system function due to their antioxidant properties (Alvandi *et al.*, 2021).

It has been reported that proteo-β-glucan, a polysaccharide derived from *Grifola frondosa* (curly grifola, leafy grifola, ram's head mushroom, ram's head, maitake), has a pronounced immunomodulatory effect (Bai *et al.*, 2022).

Impact on the pharmacokinetics of medicinal substances

Food-derived nanoparticles (FNs) may have the potential to deliver micronutrients due to their biocompatibility and health benefits. In the work of Xiaoting *et al.* (2022), hydrophilic FNs with ultra-small sizes of 1.7 nm were prepared from an aqueous extract solution of *A. auricula* by a hydrothermal method. Structural characterisation showed that Zn(II)-FNs were formed after the interaction of many functional groups, such as amino, hydroxyl and carboxyl functional groups on the surface of FNs with Zn^2. Zn(II)-FNs demonstrated better cellular compatibility than $ZnSO_4$ and zinc gluconate, with no apparent cytotoxicity at concentrations up to 75 μg/mL for normal rat kidney cells. A haemolysis rate of less than 5% was detected when the concentration of Zn(II)-FN was 5 mg/mL after incubation for 3 hours. Biodistribution experiments demonstrated that Zn(II)-FN had no obvious toxic effect after oral administration at a dose of 500 mg/kg body weight to mice. In this case, Zn(II)-FNs were present in the stomach, intestines, lungs, liver and kidneys. The results obtained by the researchers indicate that FNs obtained from a solution of the aqueous extract of *A. auricula* can act as a safe and effective Zn(II) nanocarrier.

In the research by Xiong *et al.* (2016), a natural water-soluble polysaccharide, *A. auricular* polysaccharide (AAP), was extracted and purified as a carrier for the hydrophilic drug doxorubicin hydrochloride (Dox-HCl). It involved the preparation of polyelectrolyte complex nanoparticles (PEC NPs) using electrostatic interaction between cationic chitosan (CS) and anionic AAP. The establishment of AAP-CS-NPs was confirmed by FTIR and TEM. The Dox-loaded AAP-CS-NPs were found to have a spherical morphology with an average diameter of 237.6 nm and a Dox-HCl encapsulation efficiency of 74.1%. The stability of Dox AAP-CS-NPs was tested by suspending the nanoparticles in PBS (pH 7.4) at room temperature. The particle size of the nanoparticle samples remained stable and presented no obvious changes in drug content after half a month. In addition, *in vitro* cytotoxicity studies demonstrated that the empty AAP-CS-NPs did not exhibit any cytotoxic effects, while the Dox AAP-CS-NPs enhanced the cytotoxicity of Dox-HCl against MCF-7 cells as a result of a significant increase

in cellular uptake compared to free Dox-HCl. Thus, the overall results obtained indicate that AAP-CS-NPs are very effective in capturing Dox-HCl and penetrating tumour cells, making them promising carriers for hydrophilic anticancer drugs.

A drug delivery system based on a polysaccharide biopolymer is promising (Chis *et al.*, 2022). The FA-AAP-CDDP complex can be used for the selective transport of chemotherapeutic drugs to cancer cells (Eroğlu *et al.*, 2016).

The analysis of the literature demonstrates the wide possibilities of using basidiomycetes in veterinary medicine. It can be stated that scientific interest in their research has been growing significantly in recent decades (Eroğlu *et al.*, 2016; Géry *et al.*, 2018; Zhao & Zheng, 2021), and their use in medicine is changing from empirical ideas to scientifically based knowledge (Qiu *et al.*, 2018; Alvandi *et al.*, 2020). Basidium mushrooms have been established to be effective in treating cancer (Rehman *et al*., 2020; Lu *et al*., 2021), metabolic disorders such as obesity (Liu *et al.*, 2022; Yu *et al,* 2022), diabetes (Su *et al*., 2022; Zhang *et al*., 2022), their antioxidant properties (Su & Li, 2020; Zhang *et al*., 2020; Alvandi *et al.*, 2021), efficacy in gastrointestinal diseases (Liu *et al*, 2022; Yang *et al*., 2021), diseases of the circulatory system (Wang *et al*., 2019; Bian *et al*., 2022), neurodegenerative diseases (Cordaro *et al.*, 2021; D'Amico *et al.,* 2021; Abitbol *et al.*, 2022), positive effect on the immune system (Ibe *et al*., 2020; 2021; Bai *et al*., 2022), efficacy in the treatment of burns (Buyantogtokh *et al*., 2020), antimicrobial and antiviral efficacy (Shahzad *et al*., 2020; Su *et al*., 2022), etc. Thus, Shahzad *et al*. (2020) suggested that one of the potential sources of drugs against the SARS-CoV-2 virus could be Inonotus obliquus (IO), a well-known chaga mushroom that typically grows in Asia, Europe and North America and is widely used as a raw material for various diseases around the world. Considering this, researchers have been evaluating the most effective herbs and mushrooms with antiviral and anti-inflammatory effects in the laboratory. Wei *et al*. (2018) reported on the anti-inflammatory effect of new polyoxygenated thiatanditerpenoids, neocyanins, obtained from the liquid culture of the medicinal basidiomycete *Cyathus africanus*. Subsequently, the scientists investigated 8 new highly polyoxygenated cyanotannaditerpenoids, called K-R neocytins, which were isolated from the solid culture of *C. africanus*.

A prerequisite for the development of effective medicines in veterinary medicine is their preclinical (determination of acute and chronic toxicity parameters, cumulative properties, study of irritant action, allergenic properties) and clinical studies, study of their pharmacodynamics and pharmacokinetics parameters. In this context, an analysis of the results of studies on the effect of active substances of basidiomycetes on the transport of medicinal substances presented in the literature was conducted. Such experiments were performed by Eroğlu *et al*. (2016), Xiong *et al*. (2016), Xiaoting *et al*. (2022) using nanoparticles or a polysaccharide biopolymer. Li *et al*. (2020) explored the effects of Coriolus versicolor plant polysaccharides (CVPs) on neuronal apoptosis in a rat cerebral ischaemia-reperfusion model (CIRI) and explored the mechanisms and efficacy of CVPs in the treatment of neuronal apoptosis in CIRI rats, including neurological function, brain infarct volume, inflammatory factors, etc. Bai *et al*. (2019) proved that protea-β-glucan derived from *Grifola frondosa* (PGM) can improve learning and reduce memory decline in laboratory animals by attenuating neuronal loss and histopathological abnormalities in APP/PS1 mice. Wang *et al*. (2019) explored the effect of *Coriolus versicolor* (CV) on cardiac function in diabetic rats. The protective effect of CV was determined to be associated with the inhibition

of TGF-β1/Smad signalling and attenuation of NLRP3 inflammatory activation, suggesting that CV extract may be a potential therapeutic agent. In the studies of Yang *et al*. (2021), *Hericium erinaceus* polysaccharides (HEP), which were isolated from the fruiting bodies of *H. erinaceus* (hedgehog geranium, lion's mane), it was established that, based on the results of 16S sequencing of the intestinal microbiota in adult mice, and in middle-aged and old animals, the relative abundance of *Lachnospiraceae* and *Akkermansiaceae* significantly increased, while the relative abundance of *Rikenellaceae* and *Bacteroidaceae* decreased. Bacterial solutions from different segments of the mouse intestine and faeces were collected for HEP fermentation *in vitro*. HEPs significantly promoted the production of NO, IL-6, IL-10, INF-γ and TNF-α and enhanced the phosphorylation of signalling molecules, indicating the presence of immunomodulatory activity and the possibility that HEPs could be used as active ingredients in medicinal products. Gery *et al*. (2018) confirm the reduction of glucose and insulin levels and improvement of the overall state of insulin resistance in experiments on laboratory mice. Ogbole *et al*. (2019) confirmed a pronounced anti-diabetic effect in an acute *in vivo* test.

Thus, a large number of the analysed publications indicate a high degree of scientific interest in the study of the pharmacological properties of basidiomycetes in medicine, which provides ample opportunities for their use in veterinary medicine.

Conclusions

The presented review of literary sources demonstrates the relevance of scientific research on the pharmacological properties of *Basidiomycota*, which belong to the higher fungi and have a vegetative body with branched cellular mycelium. In addition to nutrients, they contain several biologically active substances, which is a prerequisite for their use in medicine. For many years, their use has been based on observations of the effects on changes in living things that they caused. Currently, the presence of biologically active substances that can become promising active ingredients of medicines explains the motivation of scientists from different countries to explore the pharmacological properties of basidiomycetes. Previously, fungotherapy (treatment with mushrooms) was used more as an empirical treatment based on practical experience, but now the results of studies of pharmacokinetics and pharmacodynamics of active ingredients of basidiomycetes using innovative methods provide ample opportunities for the scientifically based use of this species of mushrooms in veterinary medicine. The results of scientific research by many scientists from different countries demonstrate that basidium mushrooms have a wide range of pharmacological effects. They are effective in treating oncological and neurodegenerative diseases, diseases of the digestive, respiratory and cardiovascular systems, metabolic disorders, antibacterial, antiviral (including covid infection), immunomodulatory effects, and can be used for burn treatment, etc. Their involvement in the transport of drugs at the molecular level is being actively explored using modern molecular methods. Currently, using basidiomycetes for preventive purposes and the treatment of animals with various diseases is a scientifically sound and promising area of veterinary medicine. The results of modern research identify new properties of basidiomycetes, which indicates the prospects of their multidirectional use, in particular as a source of biologically active substances with valuable pharmacological properties in oncology, non-infectious and infectious pathology and as a transport form of drugs at the molecular level, which will significantly improve the effectiveness of prevention and treatment of animals with various diseases.

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None.

Conflict of Interest

None.

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Сучасний стан наукових досліджень та перспективи використання базидіоміцетів у ветеринарній медицині: літературний огляд

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Анотація. Актуальність дослідження зумовлена необхідністю узагальнення сучасних даних про фармакологічні властивості базидієвих грибів (*Basidiomycota*) та в науковому обґрунтуванні їх застосування у ветеринарній медицині. Метою роботи було провести аналіз результатів останніх експериментальних досліджень щодо фармако-токсикологічних властивостей базидієвих грибів та можливостей їх використання у ветеринарній медицині. Для цього використовували теоретичні методи аналізу та порівняння даних літературних джерел з формулюванням обґрунтованих висновків та рекомендацій на перспективу. Базидіоміцети належать до відділу вищих грибів, їх вегетативне тіло представлене розгалуженим клітинним міцелієм. Вони є корисним продуктом харчування та багаті на біологічно активні речовини. Аналіз даних літератури засвідчує високий ступінь наукового

інтересу до вивчення їх фармакологічних властивостей. У зв'язку з цим, науковий пошук спрямовано на встановлення нових даних про фармакодинаміку і фармакокінетику біологічно активних речовин базидієвих грибів. Наприклад, гриб *Inonotus obliquus (I. obliquus)* раніше традиційно використовувався для лікування хворих за різних шлунково-кишкових хвороб. Упродовж останніх років все більше з'являється науково обґрунтованих доказів, які вказують на ефективність його екстрактів за інфекцій та інвазій, онкологічних хвороб, цукрового діабету. Встановлено, що біологічно активні речовини *I. obiquus* стимулюють імунну систему, проявляють гіпоглікемічний потенціал, чутливі до інсуліну, мають антиоксидантний вплив. У сучасному науковому дискурсі дослідники намагаються з'ясувати молекулярний механізм дії екстрактів *I. obliquus*. Представлений огляд літературних джерел засвідчує, що базидіоміцети проявляють фармакологічні властивості за онкологічних та нейродегенеративних хвороб, за хвороб органів травлення, дихання та серцево-судинної систем, за розладів метаболічних процесів, володіють протимікробною, противірусною, імуномодулюючою дією тощо. Нині у світі застосування базидіоміцетів із врахуванням їх науково обґрунтованих та експериментально доведених фармакологічних властивостей є надзвичайно перспективним. Матеріал статті становить практичну цінність для розширення можливостей застосування фунготерапії у ветеринарній медицині

Ключові слова: гриби; профілактика; хвороби; фунготерапія; біологічно активні речовини; молекулярні механізми

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Evaluation of the effect of wheat germ meal on the development of laboratory mice

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Abstract. Nowadays, dietary supplements are increasingly used in livestock feeding, which are a valuable source of biologically active substances necessary for their full growth and development, maintaining the body's resistance and preventing numerous diseases. The most common components are proteins, vitamins and carotenoids. Thus, the relevance of scientific research is to experimentally determine the effectiveness of the newly developed product with a multi-component composition on the functional state of the animal body. The purpose of the research was to determine the effect of the new product, the dietary supplement "Wheat germ meal" on the behaviour, growth rate, and morphological and biochemical parameters of the blood of white mice. The material of the study was nonlinear white mice in the amount of 60 heads. The supplement was fed to mice for 60 days. A set of methods was used, including: an assessment of the microclimate of the laboratory animal housing, the condition of tap water for drinking mice, an assessment of their general behaviour, and a determination of haematological parameters. It was proved that during the experiment, the microclimate of the room in which the laboratory animals were kept and the indicators of tap water for their drinking corresponded to the requirements of current regulations. It is substantiated that the examined additive increases the body weight of white mice and their average daily weight gain. Therewith, the weight of the internal organs of the experimental group (thymus, thyroid gland, kidneys, liver, lacrimal gland) remained unchanged. It has been established that the component composition of the supplement affects the morphological parameters of the blood of white mice, namely, it increases the haemoglobin content, the number of red blood cells and the value of haematocrit. No changes were observed in the blood leukogram of mice. An increase in the content of total protein and globulins in the blood serum of white mice was established. A decrease in the albumin content and an increase in the activity of the enzymes alanine aminotransferase and aspartate aminotransferase occurred within the reference values for white mice. The materials of the research are of practical value for the possibility of using the examined dietary supplement in domestic animals to increase muscle strength, improve growth and development, and strengthen the immune system

Keywords: housing conditions; dietary supplement; growth intensity; blood morphological parameters; biochemical parameters

Introduction

The successful development of livestock production and its specialisation allows obtaining the maximum amount of high-quality products at minimal costs (Wang *et al*., 2022). However, in the current market conditions, to find resource savings to ensure the profitability of the livestock sector, farms use the latest European animal husbandry technologies. Thus, the search for effective, harmless, environmentally friendly means to ensure the growth, development and increase of natural resistance of young animals for their high preservation is currently relevant (El-Bahr *et al*., 2020).

Using various biologically active compounds during the rearing of young animals will significantly improve the problem of their safety. For example, vitamin preparations can ensure intensive growth, development and high safety of young animals. It has been established that the protective function of probiotics and vitamin preparations is manifested by immunostimulation with increased phagocytosis activity,

synthesis of secretory IgA, interferon, increased functional activity of immunocompetent cells, induction of lymphokines, which positively affects specific and nonspecific factors of natural resistance in calves of the early postnatal period, maintaining the body's homeostasis, ensuring their normal health (Azarang *et al.*, 2020; Bayat *et al.*, 2023; Fei *et al.*, 2023). Vitamin preparations stimulate the main stages of metabolism in tissues, and contribute to the processes of restoring the functioning of vital organs (Mahdinloo *et al.*, 2022; Zaaboul & Liu, 2022).

Currently, a fairly large number of vitamin preparations have been proposed for animal husbandry, especially in industrial technologies, therefore, research on the effectiveness of their impact on the animal body, the feasibility of their use and the determination of economic efficiency of their implementation are of scientific and practical interest. Therewith, the effectiveness of biologically active preparations derived from wheat germ, which are natural medicines and catalyse cation exchange and adsorption processes, has not been sufficiently analysed. Wheat germ is known to be a highly nutritious by-product of the flour milling industry with a short shelf life. The shelf life of this product depends on the manufacturing method. Thus, the process of fermentation of different concentrations of germs reduces the activity of lipase and lipoxygenase, which are associated with the release of fatty acids (Khosroshahi *et al.,* 2022). The method of producing stabilised wheat germ by mixed fermentation (*Lactobacillus acidophilus* and *Lactobacillus plantarum*) and electrospraying process actively stabilises the enzymes (Khosroshahi *et al.*, 2023).

Since β-carotene is the main carotenoid of provitamin A found in grains such as sorghum, and when its concentration is below the target, biofortification breeding studies are being conducted (Cruet-Burgos *et al.*, 2023). The effect of vitamin preparations, including those containing carotenoids, on the body depends on the component composition and dosage. Thus, it is important to evaluate the impact of the new composition of biologically active drugs on the animal body. These studies should begin with laboratory animals. The purpose of the research – to evaluate the effect of the dietary supplement "Wheat germ meal" on the growth and development of white mice.

Literature Review

Recently, researchers have been paying more and more attention to natural vitamin preparations containing carotenoids. Significant scientific and practical interest in these compounds is explained by their ability to prevent the development of many diseases, such as cancer, neurodegenerative diseases such as Alzheimer's disease, cerebral ischaemia, diabetes associated with obesity and hypertension, ophthalmic diseases, etc. Carotenoids can be used as food additives – nutraceutical and pharmaceutical compounds. Therewith, research is required to explore the availability of these compounds to the human body (Bhatt & Patel, 2020).

Carotenoids are not synthesised in the body of animals but accumulate from feed or are partially modified as a result of metabolic reactions. The carotenoids produced in animals are diverse in structure and play an important role as precursors of vitamin A, photoprotectors, and antioxidants, promoting reproduction and strengthening the immune system (Maoka, 2020). Nowadays, about 600 carotenoid pigments have been isolated from various sources. The prevalence and diversity of carotenoids in nature are conditioned upon both the ability of living systems to biosynthesise them and their ability to adsorb and metabolise these substances. Complex vitamin compositions in different groups and species of living organisms vary both in content and composition. Natural sources of carotenoids are very diverse:

a significant amount of them are found in herbs and green leaves, flower petals, algae, and various microorganisms (Foong *et al.*, 2021).

Marine carotenoids (astaxanthin, fucoxanthin, β-carotene, lutein, and the rare siphonaxanthin, sioxanthin, and mixol) have recently demonstrated antioxidant properties in reducing oxidative stress markers (Genç *et al.*, 2020). Currently, carotenoids can be obtained from plant materials by chemical synthesis and biotechnological means (Kalra *et al.*, 2021). The synthetic production of carotenoids increases efficiency compared to the natural metabolic system and is based on the design of a three-domain enzyme (CrtB, CrtI, CrtY) that contains the complete β-carotene pathway (Rabeharindranto *et al.*, 2019). Carotenoids are of particular interest in commercial poultry farming, both for high poultry productivity and for the production of functional food products. It is known that carotenoids can accumulate in organs and tissues, stimulating weight gain in poultry. The positive effect of lycopene on metabolic processes and live weight gain in broiler chickens has been demonstrated by other authors, and their content depends on the availability of the poultry feed (Marounek & Pebriansyah, 2018).

Carotenoids – are fat-soluble compounds with subsequent digestion and absorption in the gastrointestinal tract. Their absorption begins with their release from the food matrix and dissolution in the lipid phase, and then incorporation into lipid micelles in the small intestine, which is absorbed by the mucous membrane and transported by the lymphatic system in the form of chylomicrons through the lymphatic vessels. Mostly β-carotene accumulated along the duodenal-iliac axis of the intestine and was subsequently converted to retinol and retinyl esters in its proximal section (Reboul, 2023). Natural carotenoids extracted and purified from the aquacultured marine green macroalgae *Ulva ohnoi* and freshwater green microalgae *Haematococcus pluvialis* contributed to the regulation of the gut microbiota, affecting gut homeostasis and health (Pratap *et al.*, 2022).

The accumulation of carotenoid lycopene in tissues depends on gender, genotype and tissue type. Administration of lycopene to rats for two weeks had no adverse effect on daily weight gain, the relative weight of internal organs and serum enzyme activity and total liver lysosomal enzyme activity (Bradley *et al.*, 2022). The administration of tomato oil concentrate to rats decreased the level of triglyceride accumulation in the liver (Kimura *et al.*, 2021) and led to its faster removal from tissues with a decrease in oxidative stress (Sarker *et al.*, 2021; Akbari *et al.*, 2022) and inflammation. Feeding the algae *Dunaliella bardawil* to laboratory mice as the only source of vitamin A reduced markers of adipose tissue macrophage recruitment and plasma lipid concentrations (Surman *et al.*, 2020; Melnikov *et al.*, 2022). During the development of hypercholesterolaemia in rabbits and the regression of the process, there was a decrease in serum cholesterol levels due to lycopene, accompanied by a decrease in the concentration of lipid peroxidation products in tissues (Hassan *et al.*, 2021).

Carotenoids, in particular, lycopene, were involved in the regulation of lipid metabolism in poultry. Thus, feeding 100, 200 and 400 mg/kg of lycopene to broiler chickens increased body weight, decreased abdominal fat accumulation, and decrease triglyceride and total cholesterol levels, and serum low-density lipoprotein cholesterol in the blood (Wan *et al.*, 2021; Fathi *et al.*, 2022).

Thus, the data presented indicate that using biologically active additives containing vitamin preparations of natural origin and carotenoids in animals and poultry helped to improve the clinical condition, immunological status, and biochemical and physiological parameters

of the organism, and increased the supply of fat-soluble vitamins to the animal body.

Materials and Methods

The work was performed based on the problematic laboratory of farm animal immunology of Bila Tserkva National Agrarian University in the period from 2020 to 2021. The experiment used 60 heads of laboratory animals – nonlinear white mice (males) (*Mus musculus L*), free from pathogenic microflora, which were kept with barrier elements in an improved conventional system. The animals were divided into two groups (30 animals each): control and experimental. The experimental white mice were fed the dietary supplement Wheat Germ meal daily for 60 days at a dose of 2.0 g/head once a day in combination with the main diet. The main diet of white mice consisted of freshly crushed wheat grain and compound feed. Watering was performed by a nipple drinker.

"Wheat germ meal" dietary supplement was developed by "Bilotserkivkhliboprodukt" collective enterprise, Bila Tserkva, Kyiv region. This supplement is a grey powdery substance obtained from wheat germ by alcohol extraction. The supplement contains a complex of biologically active substances, namely: amino acids: lysine, histidine, arginine, threonine, serine, glutamic and aspartic acid, proline, glycine, alanine, cystine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, B vitamins, vitamin E, PP and carotenoids (1 g of the product contains B vitamins, namely vitamin B1 – 0.16 mcg/g; B2 – 0.19 mcg/g; B12 – 0.0015 mcg/g; carotenoids 4.0 mcg/g; vitamin E – 2.0 mcg/g).

At the first stage of the study, the microclimate of the room where the white mice were kept was assessed (SOU 85.2-37-736:2011..., 2011). The temperature in the room was measured by a mercury thermometer in degrees Celsius; relative humidity, % – by a hygrometer psychrometric VIT-2 (Ukraine); air velocity, m/s – by a balloon catathermometer (Ukraine); concentration of harmful gases: carbon dioxide, %; ammonia, hydrogen sulfide, mg/m^3 – by a gas analyser UG-2 (Ukraine); artificial illumination (W/m^2), lux – by a Benetech GM1010 luxmeter (Japan). The study of indoor microclimate indicators was conducted three times (before the start and on days 30 and 60 of the study) during the research period.

The microclimate indicators in the room where research laboratory animals are kept must be within the limits that meet the requirements of the applicable documents throughout the day and regardless of the season (SOU 85.2-37-736:2011..., 2011). Failure to comply with the requirements of regulations on indoor microclimate indicators can cause overheating of laboratory animals, loss of activity, decreased appetite and resistance. These clinical signs are not permissible during experimental studies and may result in the termination of further research. During the study, the microclimate of the room where the experimental animals – white mice – were kept was monitored. The average values of microclimate parameters for the research period are presented in Table 1.

Table 1. Microclimate parameters of the room for keeping white mice

Indicators	Sanitary and hygiene standards	Actual indicator
Temperature, °C	20-24	21.3±1.14
Relative humidity, %	55.0±2.0	54.6±3.09
Air velocity, m/s	0.3	0.25±0.01
CO_2 concentration, %	0.15	0.14±0.002
NH_3 concentration, mg/m^3	10	0.05±0.02

Table 1. Continued

Indicators	Sanitary and hygiene standards	Actual indicator
H_2S concentration, mg/m^3	5	0.002±0.02
Illuminance, lux (1 m from floor)	200	196.4±10.04
Photoperiod, h (light: dark)	12:12	12:12
Air exchange (frequency/hour)	10-15	10

Source: *author's development*

According to the indicators in Table 1, the microclimate parameters of the room met the sanitary and hygienic requirements (SOU 85.2-37-736:2011..., 2011). It used natural and artificial lighting – fluorescent lamps. Ventilation in the room where the animals were kept was natural, through open doors and windows.

At the second stage of the study, the tap water used to feed the laboratory animals was evaluated. These studies were conducted at the State Enterprise "Kyiv Regional Research and Production Centre for Standardisation, Metrology and Certification" according to the methodology On the approval of State sanitary norms and rules "Hygienic requirements for drinking water intended for human consumption" (2022).

The welfare of the experimental animals depended on the quality and safety of the water they were drinking. According to the requirements of regulations, drinking water must be safe from an epidemiological standpoint, harmless in terms of organoleptic and physicochemical characteristics. Thus, it was crucial to examine these indicators for compliance with the requirements of the On the approval of State sanitary norms and rules "Hygienic requirements for drinking water intended for human consumption" (2022). The results are presented in Table 2.

Table 2. Physical, chemical and epidemiological safety indicators of tap water, n=3

Indicator	Unit of measurement	Sanitary and epidemiological standards 2.2.4-171-10	In fact
1. Organoleptic characteristics			
Odour (per t=20°C)	points	≤2	1.0±0.04
Turbidity	mg/dm^3	≤1.0	0.5±0.01
Colour	deg	≤20.0	6.0±0.03
Flavour and aftertaste	points	≤2	1.0±1.25
2. Physicochemical indicators			
Hydrogen indicator	Units pH	6.5-8.5	7.5±0.06
Total stiffness	mmol/dm^3	≤7.0	7.5±0.06
Iron total	mg/dm^3	≤0.2	0.05±0.001
Chlorides	mg/dm^3	≤250	150.0±5.6
Copper	mg/dm^3	≤1.0	
Iron total	mg/dm^3	≤0.2	
Zinc	mg/dm^3	≤1.0	0.5±0.04
Manganese	mg/dm^3	0.1	0.05±0.002

Table 2. Continued

Indicator	Unit of measurement	Sanitary and epidemiological standards 2.2.4-171-10	In fact
Sulphates	mg/dm^3	⩽250	27.6±1.5
3. Epidemic safety indicators			
Total coliforms	CFU/100 cm^3	absence	absence
Intestinal helminths	Cells, eggs, larvae, in 50 dm^3	absence	absence

Source: *author's development*

Table 2 demonstrates that for all examined indicators of quality and safety, tap water corresponded to the requirements of the current regulation (On the approval…., 2022). In the third stage of the study, the general behaviour of the animals was monitored: the "open field" test was used to measure horizontal motor activity (the number of squares crossed), vertical motor activity (the number of vertical stands) and exploratory activity (the number of times the animals peered into the "holes" – the burrowing reflex).

In addition, according to the third stage of the study, the effect of the biological activity of wheat germ meal components was assessed by the dynamics of body weight of white mice (initial body weight 14.0 g) and organ weight (thymus, thyroid gland, kidneys, liver, lacrimal gland) by weighing them on a laboratory balance HC220MS (Japan). The growth rate of white mice was calculated by the average daily growth using standard methods.

At the fourth stage of the study, the morphological parameters and leukocyte blood count and biochemical indicators of the blood serum of white mice were determined. The material for morphological and biochemical studies was peripheral blood, which was collected from white mice by decapitation with a guillotine knife under preliminary anaesthesia. Blood was taken in the morning before feeding the animals on day 60 of the research. For the assessment of morphological parameters, blood was collected in special tubes with Trilon B, and for biochemical analysis, whole blood was collected, settled and centrifuged to obtain serum. The automatic haematological analyser MINDRAY BC-2300 (USA) was used to determine red blood cell count (RBC, 10^{12}/L), haemoglobin (HGB, g/L), haematocrit (HCT, %), white blood cell count (WBC, 10^9/L), neutrophils (NEUT, %), basophils (BASO, %), lymphocytes (LYMPH, %), eosinophils (EO, %) and monocytes (MONO, %). Serum biochemical indicators were assessed using an automatic biochemical analyser AS-120 (Japan) and a test system – Global Scietific (USA).

During the research, the provisions of the "General Ethical Principles for Animal Experiments" adopted by the First National Congress on Bioethics (Kyiv, 2001) were followed. The entire experimental part of the research was conducted according to the requirements of the international principles of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Scientific Purposes (1986) and the relevant Law of Ukraine No. 3447-IV "On Protection of Animals from Cruelty" (2006, February).

The obtained digital indicators were processed statistically using the Microsoft Excel software package with the calculation of the arithmetic mean and its error (M±m), the level of reliability (P) by Student's t-test ($P<0.05$; $P<0.01$; $P<0.001$).

Results and Discussion

Changes in the behaviour of laboratory animals of the experimental group and the dynamics of

their body weight compared to the control are important indicators for assessing the impact of exogenous and endogenous environmental factors (Table 3).

Table 3. Integral assessment of behavioural parameters in white mice, M±m, n=30

Indicator	Group	
	experimental	control
Body temperature, °C	38.00±0.85	38.2±1.01
Horizontal movement activity	32.20±1.41	32.1±1.12
Vertical movement activity	17.4±1.3	17.1±1.2
"Burrowing reflex"	12.40±1.05	12.00±1.20
Integral activity	64.0±2.0	64.1±1.9

Source: *author's development*

According to the results presented (Table 3), there were no changes in body temperature, horizontal, vertical, integral activity, and the "burrowing reflex" in the animals of the experimental group compared to the control group (Labunets, 2020). Changes in body weight, average daily weight gain, and weights of some organs of experimental animals are very important indicators, the violation of which indicates the degree of pathological condition of organs and impairment of specific body functions (Table 4).

Table 4. Weight and average daily weight gain of white mice, M±m, n=30

Indicator	Start of research	Day of research		
		14	30	60
Body weight, g	14.0±0.85 14.0±0.64	15.00±0.44 16.56±0.55*▲	18.00±0.84 20.38±0.59*•	20.10±1.03 23.27±1.04*•
Average daily weight gain, mg	–	210.00±1.02 230.00±1.30**	209.00±2.72 230.0±2.04**	210.00±2.86 240.00±1.40**

Notes*: *P<0.05; **P<0.001 compared to the control group. ▲P<0.01; •P<0.001 compared to the indicator at the beginning of the research. Numerator – control group; denominator – experimental group*

Source: *author's development*

As a result of experimental researches, it was established (Table 4) that on day 14 after feeding the supplement, the body weight of mice of the experimental group increased by 10.4% (P<0.05), on day 30 – by 13.2% (P<0.05), and on day 60 – by 15.8% (P<0.05) compared to the control group. Therewith, the body weight of mice in the experimental group increased by 18.3% (P<0.01) on day 14, by 45.6% (P<0.001) on day 30, and by 66.2% (P<0.001) on day 60 compared to the beginning of the research. Animals actively ate feed both from the main diet and feed additives.

The results of researches on the increase in body weight, in particular of Japanese quail, from the consumption of natural plant components of spirulina rich in vitamin A are presented by Abdel-Wahab *et al.* (2023). In addition, improvements in liver function, total lipid profile, antioxidant parameters, and immune response have been demonstrated. Therewith, Gopal *et al.* (2023) established that experimental

administration of lutein to laboratory mice significantly reduced the weight of epididymal and abdominal adipose tissue and decreased serum cholesterol levels and low-density lipoprotein cholesterol concentrations. Another carotenoid, siphonaxanthin, significantly accumulated in the mesenteric adipose tissue of male mice, but not in the epididymal and peri-renal tissue. Thus, this carotenoid effectively regulated adipogenesis and suppressed lipid accumulation (Li *et al.*, 2015).

The results presented in Table 4 demonstrate that on day 14 of the study, the average daily weight gain of mice in the experimental group increased by 9.5% (P<0.001), on day 30 – by 10.0% (P<0.001), and on day 60 – by 14.3% (P<0.001) compared to the control group. During the entire study period, the animals of the experimental group actively ate feed.

The dynamics of the weight of the internal organs of white mice on day 60 of the experiment is presented in Table 5.

Table 5. Weight of internal organs of white mice, M±m, n=30

Internal organ	Experimental group	Control group
Thymus, mg	33.0±2.3	31.0±3.0
Liver, g	1.21±0.05	1.15±0.04
Spleen, g	0.18±0.02	0.18±0.03
Thyroid gland, mg	4.00±0.06	4.00±0.05
Kidneys, g	0.15±0.03	0.15±0.07

Source: *author's development*

According to the results obtained (Table 5), there was no statistically significant difference in the weight of internal organs of mice in the experimental and control groups.

Thus, based on the increase in body weight, average daily weight gain of mice and the weight of their internal organs, it was assumed that the growth of experimental animals was improved by increasing muscle tissue. Other researchers have found that adipose tissue hypertrophy and inflammatory processes in mice occur when there is a deficiency of β-carotenoids, an enzyme that catalyses carotenoids in the inner mitochondrial membrane (Wu *et al.*, 2021). In addition, the efficiency of β-carotene conversion to vitamin A is higher in male rats than in females (Borel *et al.*, 2021).

In addition, it is essential to evaluate the combined effect of the supplement components on different parts of haemopoiesis. Ashrafizadeh *et al.* (2022) explored *Haematococcus Pluvialis*, a green algae that is a source of astaxanthin (a beta-carotenoid compound). This compound has several biological and pharmacological properties, including antioxidant, anti-inflammatory, anti-tumour, anti-diabetic, hepatoprotective and neuroprotective effects. The authors have proven the effect of each of these properties of the supplement based on the study of the molecular mechanisms of the Nrf2 signalling pathway, which is responsible for maintaining the redox balance in the physiological state and the effect of astaxanthin on it.

In addition, Bachir *et al.* (2020) established that feeding rats with the algae *Spirulina platensis*, which contains carotenoids, leads to a slight increase in the number of leukocytes. Therewith, there was a tendency to decrease the level of neutrophils and lymphocytes.

The effect of the components of the dietary supplement "Wheat germ meal" on blood morphological indicators is presented in Table 6.

Table 6. Morphological indicators and leukocyte blood formula of white mice, M±m, n=30

Indicator	Group	
	experimental	control
Haemoglobin (HGB), g/L	94.30±1.53*	86.90±2.18
Red blood cells (RBC), 10^{12}/L	8.60±0.14**	8.00±0.12
Haematocrit (HCT), %	45.20±1.06*	41.60±1.09
White blood cells (WBC), 10^{9}/L	6.40±0.50	6.50±0.40
Nucleated neutrophils (NEUT), %	3.10±0.18	3.00±0.25
Segmented neutrophils (NEUT), %	19.80±2.60	20.10±1.80
Basophils (BASO), %	0.20±0.004	0.20±0.003
Lymphocytes (LYMPH), %	56.30±2.09	55.0±3.01
Eosinophils (EO), %	2.45±0.16	2.98±0.24
Monocytes (MONO), %	5.60±1.02	5.90±2.45

Notes: **P<0.05; **P<0.01 compared to the control group of animals*
Source: *author's development*

Using the supplement promoted the activation of erythropoiesis in the body of laboratory mice. It was demonstrated by an increase in haemoglobin level by 8.51% (P<0.05) and red blood cell count by 7.5% (P<0.01) and haematocrit by 3.6% (P<0.05).

Gunal *et al*. (2021) established that one of the functions of carotenoids, in particular lutein and zeaxanthin, is to improve mitochondrial function by reducing inflammation and activating the erythroid link of haemopoiesis. Kadek *et al*. (2021) theorised that vitamin A, E and β-carotene deficiency adversely affects calf health and increases mortality due to low levels of α-tocopherol and β-carotene. The authors proved that the paranteral administration of vitamins A and E and β-carotene to pregnant cows increased the haemoglobin content, haematocrit and red blood cell count in calves. In addition, it was established that beta-carotene is an effective antioxidant for the protection of red blood cells and has a specific effect on the level of phospholipids. It intensifies metabolic processes in the body by increasing the plasticity of cell membranes and cell membrane organoids. It is due to the antagonistic effect of phospholipids on cholesterol, which causes the stiffness and stability of the cell membrane.

It is known that white blood cells and their subpopulations are biomarkers of inflammatory reactions in the body, and their determination in the experiment is mandatory (Agbuduwe & Basu, 2020). In this study, leukocytes and their populations were determined using an automatic haematology analyser. Nowadays, it is a modern research method. For example, Hofmann & Schmucker (2021) determined the number of leukocytes using automatic differentiation, which allowed obtaining results for three or five or more populations. By measuring the volume of leukocytes, they were divided into three populations and differentiated into fractions of neutrophils and lymphocytes, and fractions of medium-sized blood cells. It is more difficult to methodically differentiate leukocytes into five populations. The authors found that by combining flow cytometry with an assessment of the physicochemical and biochemical properties of leukocytes, this result can be achieved.

As can be observed from Table 6, the number of leukocytes, neutrophils, basophils and

lymphocytes in the blood of experimental mice did not differ statistically from the number of these cells in the blood of animals of the control group. Therewith, the number of leukocytes in the blood of experimental mice decreased slightly. Shevchenko *et al*. (2021) found that feeding carotenoids such as lycopene and astaxanthin to laying hens led to a decrease in the number of leukocytes and haemoglobin content in the blood.

The state of metabolic regulation is assessed by determining the concentration of individual components. For this purpose, a biochemical analysis of blood serum was performed and the indicators that can be used to determine the state of liver and immune system functioning were determined. Studies on the effect of "Wheat germ meal" on the biochemical indicators of the blood serum of white mice on day 60 of the study are presented in Table 7.

Table 7. Biochemical indicators of blood serum of white mice, M±m, n=30

Indicators, units.	Experiment	Control
Total protein (T-Pro), g/L	66.0±1.02*	61.3±1.48
Albumin (ALB), %	51.0±2.10	58.0±3.18
Globulins (Glob), %	49.0±1.83*	42.0±2.64
Alanine aminotransferase (ALT), IU/L	112.46±14.76	102.8±16.57
Aspartate aminotransferase (AST), IU/L	161.30±15.28	158.6±17.89
Total cholesterol, mmol/L	2.0±1.1	2.0±1.2

***Notes**: *P<0.05 compared to the control group*
***Source:** author's development*

The dietary supplement examined "Wheat germ meal" is a powerful source of protein. According to the research, wheat germ has a higher protein content than flour. Iusan (2022) determined that proteins are more balanced in terms of the amino acid spectrum (close to the reference value), while flour and grains contain proteins with an unbalanced composition of essential amino acids.

The total content of proteins in the blood serum indicates the state of protein metabolism in the body of experimental mice. According to the results presented in Table 7, it was established that under the influence of a biologically active dietary supplement, the content of total protein increased by 7.7% (P<0.05) and globulins by 7.0% (P<0.05). It can be assumed that a significant increase in these indicators is the main factor in increasing the intensity of growth and development of laboratory animals. Thus, the tested additive affects the immune properties of the mice.

In addition, other researchers have found a similar effect of various carotenoids on the antioxidant status and immunity of animals. Thus, Plaza *et al.* (2020) concluded that the concentration of carotenoids (xanthophylls) correlated with the concentration of α-, β-, and γ-globulins in the blood. Aziz *et al.* (2023) substantiated a significant increase in the content of total protein and globulins, improvement of antioxidant status and immunity under the influence of carotenoids.

Lin *et al*. (2016) proved that such a powerful antioxidant carotenoid as axanthactin plays an important role in modelling the immune response. In this case, the researchers used primary cultured mouse lymphocytes both *in vitro* and *ex vivo* to assess the immunomodulatory effect of astaxanthin on cytokine production.

Treatment of mice with astaxanthin significantly increased the production of interferon and interleukin. It indicated the ability of astaxanthin to modulate the immune response of lymphocytes *in vitro* and that it partially exerts its immunomodulatory effect *ex vivo* by increasing the production of interferon and interleukin without inducing cytotoxicity.

Hassan *et al.* (2022) proved the effect of dietary supplementation with an extract of a tomato by-product containing carotenoids on increasing total plasma protein and globulins. In general, this supplementation had a positive effect on growth intensity, increased antioxidant status of the studied animals and reduced mortality.

Durge *et al.* (2022) established the importance of carotenoids for animals living in artificial conditions. Feeding these animals with lutein supplements caused an increase in superoxide dismutase activity and immunoglobulin concentration in the blood serum and a decrease in malondialdehyde and cortisol. The researchers concluded that this improved the antioxidant status and immunity and reduced stress in the experimental animals.

According to the results of the experiment, the hepatoprotective effect of the studied supplement was proved. This effect was manifested in the activation of transamination processes, as there was an increase in serum aspartate and alanine aminotransferase activity, which indicates an improvement in the energy and plastic functions of the body, the development of the main metabolic pathways of its functioning. Therewith, there was no statistically significant difference between the activity of aspartate and alanine aminotransferase in the blood of mice of the experimental and control groups.

Therewith, Shibabaw *et al*. (2019) found that an increase in hepatic marker enzymes above the reference values occurs in the event of metabolic disorders, as the liver plays a key role in regulating the metabolism of carbohydrates, lipids and proteins. However, the hepatoprotective effect of carotenoids has been proven in the case of toxic effects on the body of animals. For example, El-Beltagi *et al.* (2020) used crude spirulina extract and pomegranate juice in a rat experiment to prevent carbon tetrachloride-induced hepatotoxicity. The authors established that the infusion of a toxic substance caused a significant increase in the activity of liver marker enzymes. In the case of *Spirulina platensis* and pomegranate juice administration to animals, a decrease in the activity of liver marker enzymes was observed.

In addition, El-Baz *et al.* (2020) administered equal doses of *Dunaliella salina*, a microalgae rich in carotenoids, to rats with induced liver fibrosis. These researchers found a significant decrease in serum aspartate and alanine aminotransferase activity. As a result, the development of fibrosis, centrilobular necrosis and inflammatory cell infiltration of the rat liver was suppressed. Thus, this study of the toxic effect of wheat germ meal components proved the improvement of the functional state of the liver.

As can be observed from Table 7, there was no statistically significant difference in the content of total cholesterol in the blood serum of experimental mice with that in the control group. It indicated activation of metabolism due to anabolic processes. In general, cholesterol in the body of animals is synthesised both independently and with feed and is established during the synthesis of corticosteroid hormones, bile acids and vitamin D. It is a polycyclic lipophilic compound found in cells that affects the permeability of cell membranes. The reference limits of this component in the blood serum for white mice are 1.0-3.4 mmol/L. The results are consistent with those of Kawamura *et al.* (2021), who proved the effect of anabolic nutrient-rich foods (astaxanthin, β-carotene,

and resveratrol) on muscle mass gain. It appeared to be a consequence of activation of the mTOR/p70S6K protein signalling in muscle tissue, which increased muscle mass rather than adipose tissue.

Thus, carotenoids in the mammalian body are involved in the regulation of adipose tissue metabolism and inflammation, affect the reduction of body weight and total cholesterol levels and increase high-density lipoprotein content, as found in the study by Dutta *et al.* (2023). In addition, Cao *et al.* (2021) proved that the carotenoid astaxanthin, which is associated with the regulation of redox reactions, helps reduce oxidative stress, inflammation, and cell death, and increases lipid metabolism. Melnikov *et al.* (2022) proved that the administration of a high-fat diet to male mice and the simultaneous introduction of β-carotene led to a 30% reduction in plasma cholesterol and triacylglycerol concentrations compared to a group of mice supplemented with vitamin A alone. According to the study by Soltani *et al.* (2021) macrophages originating from specific white blood cells, monocytes, affect reducing serum cholesterol levels. They were catalysed by the components of phytochemicals, including polyphenols, alkaloids and carotenoids, which were used in preclinical studies. In other words, macrophages provided both nonspecific protection (or innate immunity) and specific protection (or acquired immunity) in vertebrates.

The effect of the examined additive on the natural resistance of animals can be explained based on the fact that it contains a complex of biologically active substances, namely amino acids, vitamins and carotenoids, which have a tonic, adaptogenic and antioxidant effect. Since the supplement contains the most carotenoids among other components – 4.0 mcg/g – it was assumed that the beneficial intestinal microflora of mice was regulated, competing with strains of opportunistic bacteria. Therewith, it is possible that conditionally pathogenic microflora was displaced from the intestinal microbial population and the development of pathogenic microorganisms was restrained. The assumptions made are consistent with the results of Yang *et al.* (2021) based on the study of the intestinal microbiota of mice. The authors have demonstrated that β-carotene increases the relative abundance of the genera *Akkermansia*, *Candidatus Stoqueficus* and *Faecalibaculum*, but reduces the relative abundance of *Alloprevotella* and *Helicobacter*. In addition, Pratap *et al.* (2022) determined that significant changes with an increase in the bacterial classes *Bacteroidia*, *Bacilli*, *Clostridia* and *Verrucomicrobia* were observed after feeding mice with ulvan and astaxanthin. Kuang *et al.* (2022) proved that high levels of beneficial bacteria such as *Clostridiaceae* were present in the intestinal microflora of sensitised mice after β-carotene administration, while the number of pathogenic bacteria such as *Streptococcaceae* decreased. The authors substantiated that after the administration of β-carotene to mice, there was a stimulation of recovery processes in the intestinal mucosa and normalisation of the intestinal microbiota.

Thus, the anti-inflammatory effect of β-carotene, its ability to regulate immune system and improve intestinal microflora of animals have been experimentally confirmed.

Conclusions

In the experimental study of the dietary supplement Wheat Germ Bran at a dose of 2.0 g/head, a positive effect of its components, in particular carotenoids, on the growth and development of white laboratory mice was proved. The microclimate parameters in the laboratory animal housing, physical and chemical parameters, and indicators of epidemiological safety of tap water corresponded to the requirements of regulations. Feeding "Wheat germ meal" at a dose

of 2.0 g/head did not cause adverse reactions in mice, promoted the activation of transamination processes, which improved the metabolism of the animal body, intensified muscle growth, and, as a result, provided an increase in body growth energy. The experimental animals consumed food and water well, and their behaviour was characterised by activity and mobility. The body weight of mice fed with the dietary supplement increased during the experiment both in comparison with the control group of animals (day 14 – by 10.4%; day 30 – by 13.2%; day 60 – by 15.8%) and with the baseline values at the beginning of the study: 14th day – by 18.3%; 30th day – by 45.6%; 60th day – by 66.2%. During the experiment, the average daily weight gain of mice increased: on day 14 – by 9.5%; on day 30 – by 10.0%; on day 60 – by 14.3%. Using the dietary supplement promoted the activation of erythrocytopoiesis. The haemoglobin level increased by 8.51%, the number of red blood cells – by 7.5% and the haematocrit value – by 3.6%. The number of leukocytes, neutrophils, basophils, lymphocytes, eosinophils and monocytes in the blood of animals fed the supplement did not change. The components of the dietary supplement increased serum total protein by 7.7% and globulins by 7.0%, and increased metabolic rate against the background of activation of anabolic processes. In the future, it is planned to explore the components of the intestinal microbiota of white mice fed this dietary supplement.

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None.

Conflict of Interest

None.

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Оцінка впливу шроту зародків пшениці на розвиток лабораторних мишей

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Анотація. Нині в годівлі свійських тварин все частіше застосовують дієтичні добавки, які є цінним джерелом біологічно активних речовин, необхідних для їх повноцінного росту та розвитку, підтримання резистентного стану організму та профілактики численних захворювань. У їх складі найпоширенішими компонентами є білки, вітаміни та каротиноїди. Тому, актуальність наукового дослідження полягає в експериментальному визначенні ефективності впливу новоствореного продукту з багатокомпонетним складом на функціональний стан організму тварин. Мета дослідження стосувалася визначення впливу нового продукту, дієтичної добавки «Шрот зародків пшениці» на поведінку, інтенсивність росту, морфологічні та біохімічні показники крові білих мишей. Матеріалом дослідження слугували нелінійні білі миші у кількості 60 голів. Добавку мишам згодовували впродовж 60 діб. Застосовували комплекс методів, які включали: оцінку стану мікроклімату приміщення з утримання лабораторних тварин, стану водопровідної води для напування мишей, оцінку їх загальної поведінки та визначали гематологічні показники. Доведено, що впродовж

експерименту мікроклімат приміщення, в якому утримувалися лабораторні тварини і показники водопровідної води для їх напування, відповідали вимогам чинних нормативних документів. Обґрунтовано, що досліджувана добавка призводить до збільшення маси тіла білих мишей та їх середньодобових приростів. При цьому, маса внутрішніх органів тварин дослідної групи (тимус, щитоподібна залоза, нирки, печінка, слезінка) залишалася без змін. З'ясовано, що компонентний склад добавки впливає на морфологічні показники крові білих мишей, а саме призводить до збільшення вмісту гемоглобіну, кількості еритроцитів і величини гематокриту. У лейкограмі крові мишей не відмічалося змін. Встановлено збільшення вмісту загального білка та глобулінів в сироватці крові білих мишей. Зниження вмісту альбумінів і підвищення активності ферментів – аланінамінотрансферази та аспартатамінотрансферази відбувалося в межах референтних значень для білих мишей. Матеріали статті становлять практичну цінність для можливості застосування досліджуваної дієтичної добавки свійським тваринам для збільшення м'язової сили, покращення росту і розвитку та зміцнення імунітету

Ключові слова: умови утримання; дієтична добавка; інтенсивність росту; морфологічні показники крові; біохімічні показники

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Microscopic changes in experimentally damaged rabbit muscles under the influence of transplanted mesenchymal stem cells

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Abstract. The relevance of the work is related to the possibility of artificially adjusting the intensity of skeletal muscle tissue regeneration in animals, which will significantly affect the tactics of rehabilitation treatment of muscle injuries in the future. The purpose of the research was to determine the effectiveness of the transplanted mesenchymal stem cells on the recovery processes in experimentally injured rabbit muscles. The method of histological examination of microscopic changes in the experimentally damaged muscle tissue of the pelvic head of the biceps femoris muscle of rabbits was used. Microscopic studies of the regeneration process of experimentally damaged striated muscle tissue established that in animals after intramuscular injection of allogeneic mesenchymal stem cells, connective tissue developed around the defect on day 2 of the experiment. On day 4, a large number of stem cells were detected in the connective tissue at the site of muscle tissue damage. On day 6, only relatively small foci of muscle tissue regeneration were observed in this area. On day 8, only small focal accumulations of stem cells were detected in this location. On the 10th day after intramuscular injection of mesenchymal stem cells, newly developed muscle fibres appeared at the site of injury. On the 14th day after intramuscular injection of mesenchymal stem cells, fully developed muscle fibres and intermuscular connective tissue were

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observed at the site of injury. In rabbits, after intramuscular injection of allogeneic mesenchymal stem cells, all regenerative processes were more active than in animals of other experimental groups and were completely completed by day 14 of the experiment. The practical value of the results obtained is to clarify the patterns of development of reparative processes in the damaged muscle area and to determine the effectiveness of using transplanted allogeneic mesenchymal stem cells in stimulating recovery processes, which is important to consider in the treatment of muscle injuries using regenerative therapy

Keywords: cell engineering; regeneration; biceps femoris; injury; skeletal muscle; histological methods

Introduction

Various sports involving animals are becoming increasingly popular in Ukraine, such as horse racing and horseback riding, bike-riding (cycling with dogs), skijoring (skiing with dogs), dog sledging, etc. Among the sports involving dogs, agility, white-pooling, dog frisbee, greyhound racing, and training of service dogs according to the international IGP testing system (a type of dog training) and mondio ring are popular.

The importance of this subject is that modern veterinary practice often encounters cases of muscle injuries among sports and working animals, namely tears, sprains and damage to the skeletal muscle tissue of the limbs. Muscle injuries are one of the most challenging problems in applied veterinary medicine, as their conventional treatment is time-consuming.

Using stem cells in the treatment of various animal diseases continues to develop rapidly. The introduction of new regenerative therapies in veterinary practice has recently attracted considerable interest (Grogan *et al.,* 2022).

Regenerative medicine is focused on restoring the functions and structure of tissues and organs using the body's biological mechanisms (El-Husseiny *et al.,* 2022).

Over the past few years, new and promising biological therapies have emerged that contribute to the therapeutic treatment of orthopaedic, inflammatory and immune processes (Dias *et al.,* 2019; Ivanovska *et al.,* 2022).

The main area of cell therapy is allogeneic mesenchymal stem cells (MSCs), which are characterised as multipotent cells of non-haematopoietic origin with the ability to self-renew and present in connective tissue (De Lima Santos *et al.,* 2019).

Stem cells – undifferentiated cells with a high capacity for self-renewal and development into tissue cells with specific functions. Thus, they are an effective therapeutic tool for improving the body's ability to repair and rebuild damaged tissues (Grogan *et al.,* 2022; McKenzie *et al.*, 2022).

Currently, mesenchymal stem cells are the most promising type of stem cells for therapeutic applications due to the ease of their isolation and cultivation, and the absence of ethical issues associated with their further use. They are becoming increasingly well-known in veterinary medicine due to their exceptional immunomodulatory properties (De Lima Santos *et al.,* 2019; Grogan *et al.,* 2022; Voga & Majdic, 2022).

The scientific literature is increasingly discussing, analysing and summarising the results of studies designed to apply allogeneic mesenchymal stem cells (MSCs) as a regenerative therapy in veterinary practice, both in experimental animals and in clinical cases (Dias *et al.,* 2019; Grogan *et al.,* 2022; Voga *et al.,* 2022).

Thus, the study and analysis of the effectiveness of innovative and promising treatment of animals with damaged muscle tissue using cellular regenerative therapy methods are quite relevant, as it can significantly accelerate the time and process of recovery of animals after injury, compared to conventional methods of treating injuries and avoiding side effects of treatment.

In experimental studies, many animal models have been used to assess the safety and efficacy of cell therapy. They were used mainly at the preclinical level to determine the prospects for further application of such methods for animal treatment (Dias *et al.,* 2019; Prišlin *et al.,* 2022). Intensive research is currently underway to expand their use in clinical settings.

Even if the mechanism of action of MSCs is not fully understood, it is known that they can interact with various types of immune cells, including T and B lymphocytes, natural killer cells, macrophages, neutrophils, and monocytes (Dias *et al.,* 2019; Prišlin *et al.,* 2022). Mesenchymal stem cells influence the innate immune system (Hoffman & Dow, 2016; Voga & Majdic, 2022).

The purpose of the research was to determine the effectiveness of mesenchymal stem cells in the restoration processes of experimental damage to the pelvic head of the biceps femoris muscle in rabbits.

Literature Review

Mesenchymal stem cells can self-renew (Romero *et al.*, 2021; Voga *et al.,* 2022) while maintaining their multipotency, as described in numerous scientific publications (Dias *et al.,* 2019). It allows obtaining the required number of cells for further clinical use, in particular, for the treatment of various diseases (Shah *et al.,* 2018; Arnhold *et al.,* 2019). For the extraction of these cells, various tissues of the adult body are most often used (Almeida *et al.,* 2010), such as bone marrow, umbilical cord, adipose tissue, placenta, and less often – amnion, tooth pulp, periosteum, etc (Kern *et al.,* 2006).

An important property of MSCs, which have been used to enhance tissue regeneration and treat animals with various diseases, is their pleiotropy, such as antiapoptosis, angiogenesis, growth factor production, antifibrosis, and the ability to migrate to the site of injury, in particular through chemotaxis (Arnhold *et al.,* 2019; Dias *et al.,* 2019).

In veterinary medicine, MSCs derived from bone marrow and amniotic fluid are often used (Barretto *et al.,* 2014; Harman *et al.*, 2016). However, the procedure for obtaining these cells usually requires surgical intervention using general anaesthesia, and, accordingly, compliance with asepsis and antisepsis rules.

Other researchers have conducted an objective macroscopic and histological assessment of the healing process of acute lesions of the rectus femoris muscle using adipose-derived stem cells. The study was conducted on 18 pelvic limbs in New Zealand rabbits, which were divided into three groups (Oh *et al.,* 2014). The results demonstrated that the addition of MSCs was accompanied by a decrease in the number of inflammatory cells in the two-week assessment (from 164.2 to 89.62 cells). Therewith, qualitative analysis of red picrosinium slides identified an increase in orange/yellow fibres in these animals, indicating the final healing process. The macroscopic evaluation demonstrated no difference between the groups. In conclusion, it is argued that using MSCs in the treatment of acute muscle injury can achieve histological advantages compared to their non-use.

However, using MSCs in clinical settings is still a challenge. Restrictions on their use in various diseases and the specifics of their application based on the type of animal hinder the development of cell-based therapies. In this research, the authors considered the possibilities of using MSCs as a powerful biopharmaceutical

agent in veterinary cellular regenerative therapy based on the available evidence, analysing the already approved experimental material of veterinary colleagues and based on the results of their research.

Materials and Methods

During the period from 2019 to 2021, experimental studies were conducted at the Centre for Cellular Technologies in Veterinary Medicine at the Academician I.O. Povazhenko Department of Surgery and Pathophysiology, which is part of the Faculty of Veterinary Medicine of the National University of Life and Environmental Sciences of Ukraine.

Using and maintaining animals for experiments complied with the requirements of the regulations of Ukraine and Directive 2010/63/EU "On the protection of animals used for scientific purposes" (Directive 2010/63/EU..., 2010), and general ARRIVE guidelines. All necessary permissions for the experiments were obtained from the Bioethics Committee of the NUBiP of Ukraine.

The experimental rabbits were kept in a separate room of the inpatient department of the Department of Surgery and Pathophysiology named after Academician I.O. Povazhenko. Each animal was assigned an individual number and kept in a separate cage. The rabbits were provided with a comprehensive diet, including "Purina" granular feed and hay. They had constant access to clean water from automatic drinkers. Sterile sawdust was used as bedding. Air exchange in the room and cage cleaning was performed twice a day. The air humidity was approximately 70-80%. The room temperature was maintained between 21 and 23 degrees Celsius. The experimental rabbits were exposed to normal conditions of light and darkness, consisting of a 12-hour cycle (Antosyuk, 2017).

Clinically healthy rabbits of the Butterfly breed were used for the study, namely: males in the amount of 105 heads, 3 months old, with a body weight of 2500-3000 kg. All animals were vaccinated with the combined prophylactic vaccine Pestorin Mormix, which is effective against rabbit myxomatosis and viral haemorrhagic disease, before the start of the study (Antosyk, 2017).

Allogeneic MSCs were cultivated according to the guidelines "Obtaining a fraction of rabbit bone marrow mononuclear cells with high proliferative activity" developed by the staff of the Department of Surgery and Pathophysiology named after Academician I.O. Povazhenko of the NUBiP of Ukraine. These cells were extracted from the red bone marrow of donor rabbits. For the extraction of red bone marrow from small animals, a method of lifetime obtaining was used, which included its selection in the area of the proximal and distal epiphyses of the humerus and femur. After shaving the hair and anaesthetising the tissues by sedating the animals, the skin was treated with a 5% iodine solution in the area of the proximal and distal epiphyses of the respective bones. At the next stage, bone marrow was aspirated using a medical needle for diagnostic puncture and spinal anaesthesia with a "Pencil" cut with a mandrel.

Cell manipulation and isolation procedures with cellular material were performed in a box of the Class II Biological Safety Training and Research Laboratory (ESCO). Further cultivation of cells was conducted in a CO_2 incubator (HERA CELL, Germany), which provided absolute humidity and 5% CO_2, and a room temperature of 37°C.

Laboratory tests were conducted in two locations: the Bald Veterinary Laboratory (Kyiv) and the Vetmedservice Training and Research Clinical Centre (NUBiP of Ukraine, Kyiv).

To determine the number of cells, a Goryaev camera and a PrimoVert microscope (Germany) with a 200x magnification were used. The total number of cells was calculated using the formula (Mazurkevich *et al*., 2010).

Cells were frozen in a Dewar vessel with liquid nitrogen (SDS-20, Ukraine). Cells were thawed in a water bath (EL 20, Poland) at 37°C.Culture media, solutions and preparations were stored in a Nord refrigerator (Ukraine) at 4°C and -18°C. Cell suspensions were centrifuged using a centrifuge (UNICO, USA). A thermostat TS-80M (Ukraine) was used to heat the solutions. Laboratory glassware was dried and sterilised in a dry heat oven NS-62A (Poland) and an air steriliser GPO-50 (Ukraine).

Before the operation with experimental reproduction of muscle tissue damage, rabbits were kept on a fasting diet for 12 hours. The operation was performed under general anaesthesia by intramuscular injection of a 2% solution of Xyla at a dose of 0.15 mL/kg of animal body weight. The rabbit was fixed on the operating table, and the access to the surgical field was freed by shaving the hair with an electric clipper and treated with Coutasept F solution.

Before the rabbit was put into general anaesthesia, an anticholinergic agent that blocks peripheral cholinergic systems was administered intramuscularly, and atropine sulfate at a dose of 0.05 mg/kg of animal body weight was used to prevent bronchospasm and laryngospasm, reduce glandular secretion, reflex reactions, and side effects caused by vagus nerve excitation. Then, "Zoletil" 100 was administered intramuscularly at a dose of 8 mg/kg of animal body weight.

Before recreating the muscle tissue injury, infiltration anaesthesia was performed at the access site with 0.5% novocaine solution (dose 3 mL/head) in the planned part of the modelled defect. The muscle tissue injury was reproduced by dissecting the skin and fascia, and then a 1.5x1.5 cm x1.5 cm section of the pelvic head of the biceps femoris muscle was cut off in the midplane area, as demonstrated in Fig. 1.

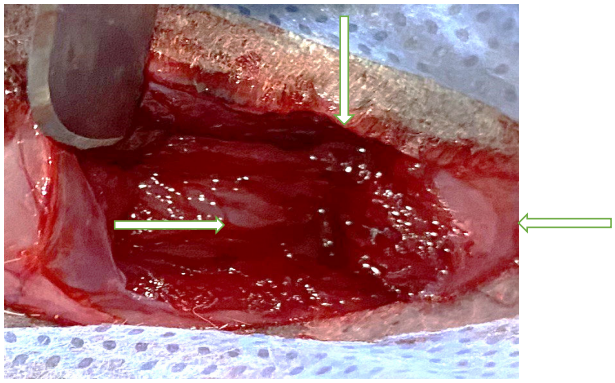

Figure 1. General view of the modelled muscle tissue defect
***Notes**: indicated by arrows; damage size 1.5x1.5 cm, 1.5 cm deep)*

The animals in the third experimental group were prescribed conventional treatment of the injury, which included two methods:

1.Surgical method: the tear in the muscle tissue was sutured with two knotted sutures. In addition, the fascia and skin were sutured with synthetic absorbable suture material Vicryl (Vicryl, Belgium). The muscle tissue was sutured with absorbable material (Chirasorb braided) (Sun *et al.*, 2018).

2. Medical method: after wound closure, 0.5 mL of Dermabond surgical glue was applied to the wound site. In addition, antibiotics were administered intramuscularly: ceftriaxone at a

dose of 0.2 mL/kg body weight once daily for 5 days and tylosin 5% at a dose of 6 mL/animal once daily for 5 days (Sun *et al*., 2018).

Animals in the fourth experimental group (control) were injected intravenously with a 0.9% sodium chloride solution. Additionally, a fifth group was established – intact animals.

After the operation, the rabbits were provided with complete peace of mind, proper housing, feeding and watering conditions. Clinical examination of animals was conducted throughout the study period.

Samples of biological material for laboratory studies were taken at baseline (before the experiment) and on days 4, 7, 10, 14, 21 and 28 of the experiment.

To obtain blood samples for further laboratory testing, a jugular vein was punctured and fresh blood was collected in tubes with an anticoagulant (Mazurkevich *et al*., 2010).

At a specific stage of the experiment, 3 animals from each experimental group were euthanised after deep anaesthesia. It was done to obtain samples of blood and muscle tissue for laboratory tests (Kern, 2006; Mazurkevich *et al*., 2010).

In the veterinary laboratory "Bald" (Kyiv), biochemical studies were performed by the spectrophotometric method using a biochemical analyser RT-9600 (China). For this purpose, commercial kits from the company "Filisit-Diagnostics" were used according to the attached instructions. Histological studies were performed at the Kasyanenko Department of Anatomy, Histology and Pathomorphology of Animals at the National University of Life and Environmental Sciences of Ukraine.

For fixation of the muscle samples, 10% formalin was used, and then the samples were dehydrated in alcohols of increasing concentration. The material was embedded in a homogenised paraffin mixture from Histomix. A sledge microtome MS-2 (Ukraine) with a section thickness of 10 and 15 μm was used to cut the blocks, according to previous studies, in particular Kern *et al.* (2006), Silva *et al*. (2010) and Sun *et al*. (2018).

For staining muscle tissue, the Van Gieson method with Karatzi's haematoxylin and eosin was used, and the preparations were placed in polystyrene. Then, the preparations were examined under an Axioskop-40 microscope (Carl Zeiss, USA) with a total magnification of 50, 100 and 400 times. Photographs of the preparations were taken using an SEO digital camera.

Morphometric parameters, such as muscle fibre thickness and interfibrillar space, were measured using the VideoTest Morphology 5.0 software. Statistical analysis of all the results was performed using the methods of analysis of variance in Statistica 10.0 software from Statsoft Inc.

Student's t-test was used to assess statistically significant differences between sample means. Differences with $P<0.05$ were considered statistically significant, which is a common practice in biological research (Mazurkevich *et al.,* 2010; Sun *et al*., 2018).

Results and Discussion

Histological studies of the regeneration of experimentally damaged striated muscle tissue in animals of experimental group 1 after intramuscular injection of allogeneic MSCs showed that on day 2 of the experiment, connective tissue was already developing around the experimentally established defect (Fig. 2). Stem cells were detected in this tissue, and in some places, a small number of these cells (2 to 4) were fused.

Evidently, intramuscular injection of stem cells enters the site of injury to a greater extent than intravenous injection, and their number in the injured area is significantly higher after local injection than after intravenous injection. In addition, significant activation of regenerative processes is documented by the presence of a large number of red blood cells in the defect area. It is assumed that the latter locally

provides significant oxygen requirements for actively functioning cells.

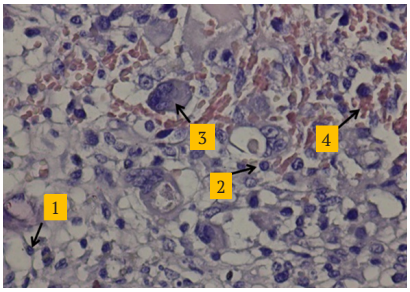

Figure 2. Average part of the experimentally developed defect on day 2 after intramuscular injection of allogeneic MSCs

Notes: *1 – fibrocyte; 2 – stem cell; 3 – stem cell fusion; 4 – normochromic red blood cells. Karatsi hematoxylin and eosin, ×400*

On day 4 of the experiment, a large number of stem cells were found in the connective tissue between muscle fibres and the establishment of new muscle fibres at the site of injury. However, a small number of partially destroyed muscle fibres were still found locally (Fig. 3).

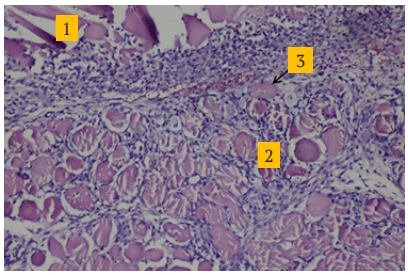

Figure 3. Average part of the experimentally developed defect on day 4 after intramuscular injection of allogeneic MSCs

Notes: *1 – partially destroyed muscle fibres; 2 – stem cells in fibrous connective tissue between muscle fibres; 3 – development of a new muscle fibre. Karatsi hematoxylin and eosin, ×50*

On the 6th day of the experiment, only relatively small foci of muscle tissue regeneration were detected at the site of injury. Most of the artificially established defect at this time was already filled with newly developed, well-developed muscle fibres, although in some places between the muscle fibres, there were still clusters of stem cells (Fig. 4).

On the 8th day of the experiment, only small focal clusters of stem cells were found at the site of injury, in which the processes of establishment of new muscle fibres were still observed. The entire area of the injury was filled with newly developed muscle tissue, which was still unevenly swollen, and the sarcoplasm of muscle fibres was pale and unevenly stained with eosin. The nuclei were dominated by heterochromatin (Fig. 5).

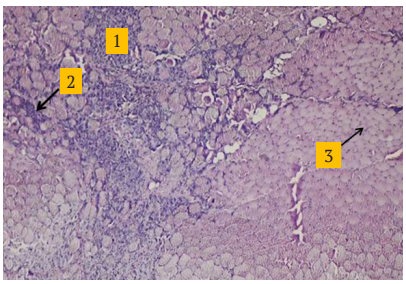

Figure 4. Average part of the experimentally developed defect on day 6 after intramuscular injection of allogeneic MSCs

Notes: *1 – focus of muscle tissue regeneration; 2 – stem cells between muscle fibres; 3 – newly developed muscle tissue. Karatsi hematoxylin and eosin, ×50*

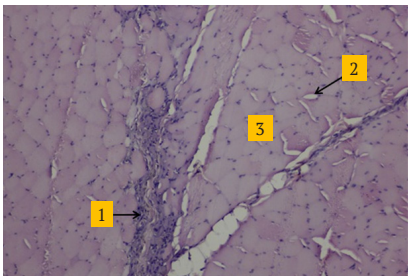

Figure 5. Average part of the experimentally developed defect on day 8 after intramuscular injection of allogeneic MSCs

Notes: *1 – focus of stem cell accumulation; 2 – oedema; 3 – newly developed muscle tissue. Karatsi hematoxylin and eosin, ×50*

On the 10th day after intramuscular injection of stem cells, only newly developed muscle fibres were detected at the site of injury. However, some of them were still incompletely developed. The muscle tissue, in general, did not vary from that of intact animals but was still slightly swollen (Fig. 6).

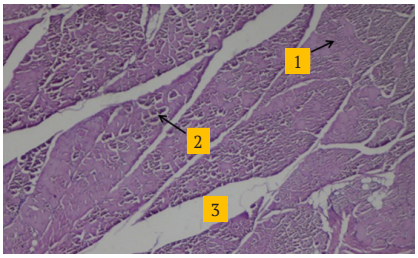

Figure 6. Average part of the experimentally developed defect on day 10 after intramuscular injection of allogeneic MSCs
Notes: *1 – established newly developed muscle fibres; 2 – incompletely established newly developed muscle fibres; 3 - oedema. Karatsi hematoxylin and eosin, ×50*

On the 14th day after the intramuscular injection of stem cells, fully developed muscle fibres and intermuscular connective tissue were observed at the site of injury, and the degree of oedema was significantly reduced compared to the previous stage of the study (Fig. 7).

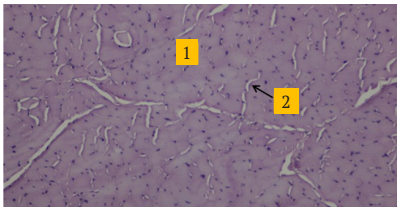

Figure 7. Average part of the experimentally developed defect on day 14 after intramuscular injection of allogeneic MSCs
Notes: *1 – newly developed muscle fibres; 2 – oedema. Karatsi hematoxylin and eosin, ×50*

Thus, in animals of experimental group 1, after intramuscular injection of allogeneic MSCs, all regenerative processes were more active compared to those in animals of other experimental groups and were completely completed by day 14 of the experiment.

In animals of experimental group 2, which were injected with allogeneic MSCs intravenously, only newly developed connective tissue was detected around the site of injury on day 2. The defect itself at this time was noticeably narrower than in animals of the control group and the group of rabbits under conventional treatment, and did not contain destroyed tissue and haemorrhages in the area surrounding the defect (Fig. 8). Instead, some of the muscle fibres around the newly developed connective tissue were destroyed, and the muscle tissue itself was slightly swollen.

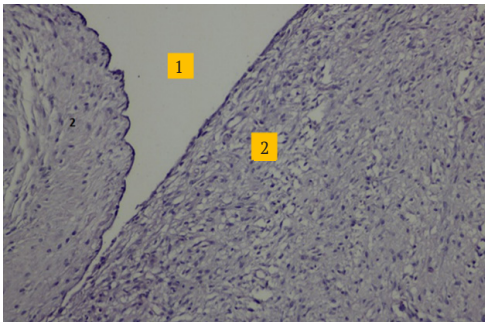

Figure 8. Upper part of the experimentally established defect on day 2 after intravenous injection of allogeneic MSCs
Notes: *1 – defect site; 2 – newly developed connective tissue. Karatsi hematoxylin and eosin, ×50*

The newly developed connective tissue contained a large number of fibrocytes, fibroblasts and collagen fibre bundles, which designed a fairly dense network (Fig. 9).

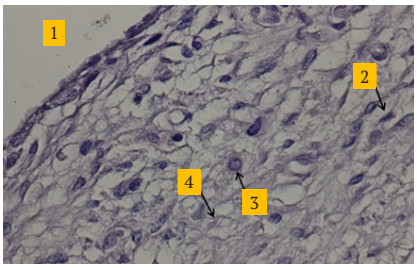

Figure 9. Upper part of the experimentally established defect on day 2 after intravenous injection of allogeneic MSCs

Notes: *1 – defect site; 2 – fibroblast; 3 – fibrocyte; 4 – collagen fibre bundles. Karatsi hematoxylin and eosin,×50*

On day 4, when stem cells were injected intravenously, small clusters of stem cells were found in the damaged and swollen muscle tissue around the defect site (Fig. 10). These stem cells were differentiated from satellite cells and leukocytes, which could theoretically infiltrate the connective tissue between muscle fibres. Satellite cells had oval or spindle-shaped nuclei and were localised on the surface of the muscle fibre. Stem cells were located in the connective tissue between the muscle fibres and were not associated with these fibres.

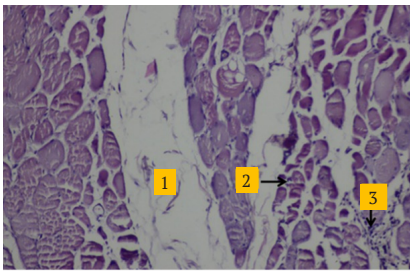

Figure 10. Average part of the experimentally established defect on day 4 after intravenous injection of allogeneic MSCs

Notes: *1 – oedema; 2 – damaged muscle fibres; 3 – stem cells in the swollen connective tissue between damaged muscle fibres. Karatsi hematoxylin and eosin, ×50*

Stem cells differed from leukocytes in their structure. They had small, intensely coloured nuclei and an extremely narrow cytoplasmic rim, which is typical of undifferentiated cells. Unlike lymphocytes, their nuclei were polymorphic (Fig. 11).

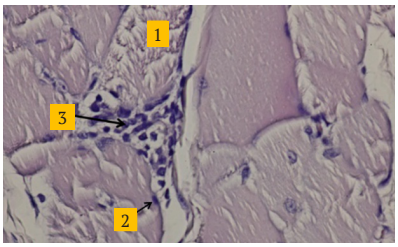

Figure 11. Average part of the experimentally established defect on day 4 after intravenous injection of allogeneic MSCs

Notes: *1 – damaged muscle fibre; 2 – satellite cell; 3 – stem cells. Karatsi hematoxylin and eosin, ×50*

On the 6th day after the injury, intravenous injection of stem cells in the area of injury demonstrated an increase in the number of stem cells in the connective tissue between muscle fibres and the presence of distinct processes of muscle tissue regeneration (Fig. 12).

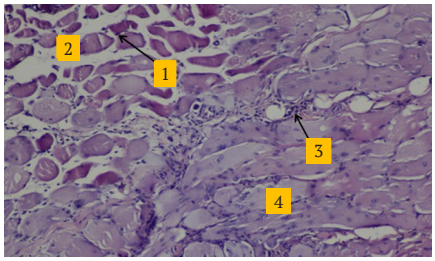

Figure 12. Average part of the experimentally established defect on day 6 after intravenous injection of allogeneic MSCs

Notes: *1 – damaged muscle fibres; 2 – oedema in the area of damaged muscle fibres; 3 – stem cells in the connective tissue between muscle fibres. Karatsi hematoxylin and eosin, ×50*

Histological studies demonstrated the process of muscle tissue development by stem cells. Initially, these cells fused to develop syncytia with a common cytoplasm. In cross-sections, these structures were round and oval in shape. Then there was a gradual increase in the volume of the common cytoplasm (Fig. 13).

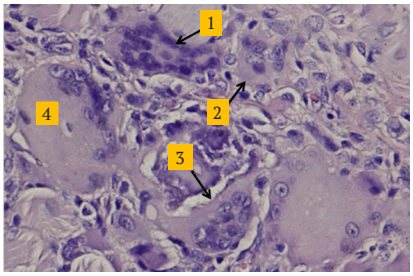

Figure 13. Average part of the experimentally established defect on day 6 after intravenous injection of allogeneic MSCs

Notes: *1 – a fusion of stem cells to establish an oval-shaped structure; 2 – a fusion of stem cells to establish a rounded structure; 3 – the initial stage of cytoplasmic volume accumulation in the cell syncytium established by stem cell fusion; 4 – a distinct increase in the volume of the common cytoplasm in the structure established by stem cell fusion. Karatsi hematoxylin and eosin, ×50*

The longitudinal section demonstrates that thin fibres of muscle-like tissue are established at first, followed by a distinct thickening of these newly developed fibres, and satellite cells appear on their surface (Fig. 14).

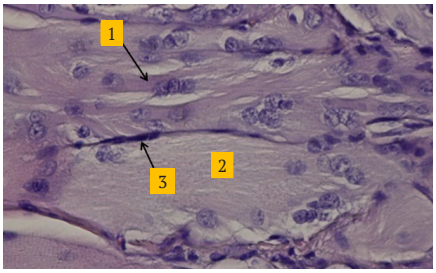

Figure 14. Average part of the experimentally established defect on day 6 after intravenous injection of allogeneic MSCs

Notes: *1 – thin fibre, similar to muscle tissue; 2 – distinct thickening of the newly developed fibre; 3 – satellite cell. Karatsi hematoxylin and eosin, ×400*

However, at this stage of the regenerative process, the newly developed fibres were still undifferentiated – they did not have the microscopic structure characteristic of striated muscle tissue.

On the 8th day after the injury, intravenous injection of stem cells in the area of injury demonstrated the presence of a large number of newly developed muscle fibres and a clear infiltration of connective tissue layers between the newly developed muscle fibres by stem cells. Cellular syncytia, which occurred on day 6 after the injury, were no longer detected. These changes indicated a high rate of the regenerative process (Fig. 15). However, the sarcoplasm of the newly developed muscle fibres still had a heterogeneous density, was vacuolated in some places, transverse striations were not detected, and heterochromatin significantly prevailed in nuclei, which indicated intensive processes of substance synthesis, i.e., the continuation of the processes of muscle fibre development (Fig. 15).

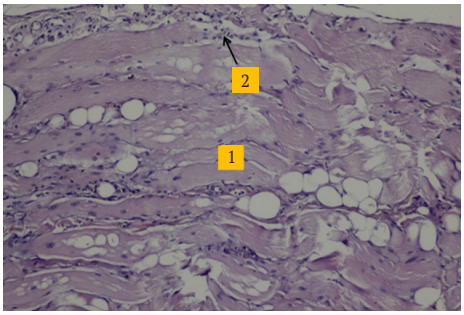

Figure 15. Average part of the experimentally established defect on day 8 after intravenous injection of allogeneic MSCs

Notes: *1 – newly developed muscle fibres; 2 – stem cell infiltration of connective tissue layers between newly developed muscle fibres. Karatsi hematoxylin and eosin, ×50*

On the 10th day after the injury with intravenous injection of stem cells, microscopic changes in the area of injury were similar to those on the 8th day, except for the absence of sarcoplasmic vacuolation of the newly developed muscle fibres.

On the 12th day after the injury, only small foci of the regenerative process and a small number of incompletely developed muscle fibres were detected in the area of injury after

intravenous injection of stem cells (Fig. 16). The newly developed muscle fibres still had sarcoplasm of heterogeneous density.

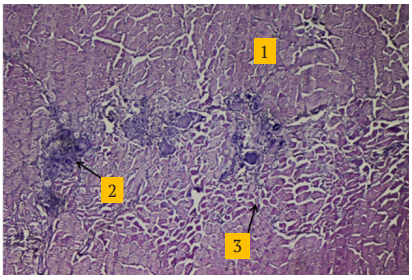

Figure 16. Average part of the experimentally established defect on day 12 after intravenous injection of allogeneic MSCs

Notes: *1 – newly developed muscle tissue; 2 – regeneration focus; 3 – incompletely developed muscle fibres. Karatsi hematoxylin and eosin, ×50*

On the 14th day after the injury, only muscle fibres were detected in the area of injury after intravenous injection of stem cells. However, they still had sarcoplasm of heterogeneous density, and the number of satellite cells on their surface was increased (Fig. 17). Such changes indicated incomplete development of striated muscle fibres in the area of injury. However, between the bundles of muscle fibres, layers of fibrous connective tissue typical for skeletal muscles, in which blood vessels passed, had already been established.

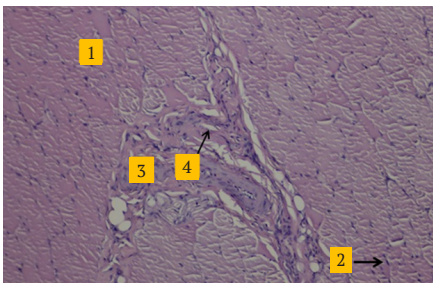

Figure 17. Average part of the experimentally established defect on day 14 after intravenous injection of allogeneic MSCs

Notes: *1 – newly developed muscle fibres; 2 – satellite cells; 3 – fibrous connective tissue layer; 4 – blood vessel. Karatsi hematoxylin and eosin, ×50*

The results of the author's research are compared and analysed with the most recent research conducted by a group of researchers in 2022, which demonstrates the promise of using various methods of treatment applications in veterinary medicine, such as stem cells, in the development, improvement and application in veterinary practice.

In research (Santos *et al.*, 2022), in an experimental model of acute muscle injury, it was suggested that allogeneic MSCs can be used to optimise the muscle healing process through three mechanisms: 1) production of growth factors to promote angiogenesis and prevent cell apoptosis; 2) immunosuppressive effect to reduce the activity of T- and B-lymphocytes; and 3) inducing fibroblasts to differentiate into myocytes. The authors of the publication chose acute muscle injury with early evaluation as stem cells demonstrate greater functionality and productivity in the first days after administration, which is consistent with the results of the evaluation presented in the described work.

Thus, the researchers hope to determine the possible acceleration of functional recovery after using allogeneic MSCs. An important originality of the research is that it is the first study to explore using allogeneic MSCs to treat acute muscle injury in an experimental model. This work is a continuation of the study on using allogeneic MSCs for the treatment of acute muscle injury in an experimental model.

Limitations of this research include difficulties in sampling, as this is a groundbreaking study; using an experimental model that does not fully reflect clinical practice, as acute cut muscle injuries are rare; and the lack of additional evaluation by other methods.

The results of this research emphasise the importance and potential of using allogeneic mesenchymal stem cells in the field of veterinary medicine, in particular, in the treatment of acute muscle injury. Analysing the most re-

cent similar studies (El-Husseiny *et al.*, 2022; Grogan *et al.*, 2022; Santos *et al.*, 2022) and based on the above, it becomes obvious that using MSCs in muscle tissue injury is a promising area of modern veterinary medicine. Allogeneic bone marrow-derived mesenchymal stem cells, collected from recipient animals, act as an effective stimulator of femoral muscle myocyte regeneration.

An inflammatory muscle healing process was observed in both study groups, indicating the presence of inflammatory tissue in the sample. However, the results of the quantitative analysis demonstrated that the addition of ADSC caused a decrease in the number of inflammatory cells per field at the 2-week assessment. The quantitative analysis demonstrated a decrease from 164.2 cells per field in the group without ADSC supplementation to 89.62 cells per field in the group with ADSC supplementation, representing a 46% reduction in the number of inflammatory cells after ADSC supplementation. Considering that the control group did not demonstrate signs of inflammation, this quantification was not performed for the control group.

The author's research has established that using allogeneic MSCs in the repair of damaged muscles helps to accelerate the regenerative process by replacing damaged areas with muscle cells. It was confirmed by microscopic analysis of histological specimens, which demonstrate these changes.

Among other things, it was established that the speed and outcome of the recovery of experimentally damaged muscle tissue in rabbits depends on the method of allogeneic MSCs administration. Injections of cells into the muscles directly (intramuscular injections) demonstrated better results than injections through the vein, and complete recovery was observed on day 14 of the experiment. When cells were injected through a vein after muscle tissue damage, only muscle fibres with incomplete maturity were observed in the area of injury, which had an increased number of satellite cells on the surface. It indicates the incomplete process of fibre development of striated muscle tissue in the injured area. However, typical layers of fibrous connective tissue with blood vessels, characteristic of skeletal muscle, were already present between the fibres. The intravenous administration of stem cells led to a significant acceleration of the recovery of damaged muscles, which coincides with the results of other researchers. However, the activity of regenerative processes was lower than in animals of experimental group 1, which were injected with allogeneic MSCs intramuscularly.

The research results are of great practical importance for the further development and implementation of cell therapy in veterinary medicine, in particular for the effective treatment of muscle tissue injuries in animals, particularly in the case of heavy physical exertion on skeletal muscle and when used in sports competitions. These results demonstrate the potential of cell therapy to improve muscle recovery and may serve as a foundation for the further development of new methods of treating muscle injuries in veterinary practice. Using allogeneic MSCs in clinical practice may have broad prospects for the treatment of humans with muscle damage. These studies may contribute to the development of new therapeutic approaches and open up opportunities for rapid and effective muscle recovery after injuries and in the development of myopathies.

Further research and clinical trials may provide new perspectives in the field of regenerative medicine and contribute to the development of new methods of treating muscle injuries in veterinary medicine.

Conclusions

By modelling the experimental injury of the pelvic head of the biceps femoris muscle in

rabbits, research was conducted to determine the effectiveness of mesenchymal stem cells in terms of the intensity of recovery processes with different methods of their administration (at the site of the experimental defect and intravenous administration), and comparison with the conventional method of treatment. Modelling of experimental muscle tissue damage was performed by dissecting the skin and fascia and cutting off the biceps femoris in the area of the midplane of the pelvic head, measuring 1.5x1.5 cm to a depth of 1.5 cm of muscle tissue.

As a result, it was established that using allogeneic mesenchymal stem cells to stimulate the recovery of experimentally damaged muscles significantly accelerated the recovery of the damaged area by filling the defect with full-fledged muscle cells, which was confirmed microscopically using the histological method of examination.

Therewith, it was determined that the rate and order of restoration of experimentally damaged muscle tissue in experimental rabbits using transplanted allogeneic MSCs depended on the method of their administration. In particular, local (intramuscular) injections of allogeneic MSCs demonstrated better results than intravenous injections and were completely completed by day 14 of the experiment. Therewith, in the case of intravenous injection of stem cells after experimental muscle tissue injury, only muscle fibres were detected in the area of injury, which still had sarcoplasm of heterogeneous density and an increased number of satellite cells on their surface, indicating incomplete formation of striated muscle fibres in the area of injury. However, between the muscle fibre bundles, layers of fibrous connective tissue typical of skeletal muscles with blood vessels were already established.

The results of the experimental studies are of great practical importance for the further development and implementation of cell therapy in clinical veterinary practice, including as a highly effective method of treating muscle tissue injuries in animals, particularly in cases of intense physical activity on skeletal muscle and when used in sports competitions.

In the future, it is planned to conduct comparative studies and clinical trials on the effectiveness of animal cell therapy in the case of spontaneous muscle damage to develop appropriate treatment protocols for similar forms of myopathy and their widespread further implementation in applied veterinary medicine.

Acknowledgements

None.

Conflict of Interest

None.

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Мікроскопічні зміни в експериментально ушкоджених м'язах кролів за впливу трансплантованих мезенхімальних стовбурових клітин

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Анотація. Актуальність роботи пов'язана із можливістю штучно коригувати інтенсивність регенерації скелетної м'язової тканини у тварин, що на перспективу істотно вплине на тактику відновлювального лікування м'язових травм. Мета наукового дослідження полягала у визначенні ефективності впливу трансплантованих мезенхімальних стовбурових клітин на відновлювальні процеси в експериментально ушкоджених м'язах кролів. У роботі використано метод гістологічного дослідження мікроскопічних змін в експериментально ушкодженій м'язовій тканині тазової голівки двоголового м'яза стегна кролів. Завдяки мікроскопічним дослідженням процесу регенерації експериментально ушкодженої посмугованої м'язової тканини встановлено, що у тварин після внутрішньом'язового введення алогенних мезенхімальних стовбурових клітин вже на 2 добу досліду навколо створеного дефекту формувалася сполучна тканина. На 4 добу в місці пошкодження м'язової тканини виявляли велику кількість стовбурових клітин у сполучній тканині. На 6 добу в цьому місці відзначали лише відносно невеликі вогнища регенерації м'язової тканини. На 8 добу в цій локації виявлялися лише невеликі вогнищеві скупчення стовбурових клітин. На 10 добу після внутрішньом'язового введення мезенхімальних стовбурових клітин у місці пошкодження з'являлися новоутворені м'язові волокна. На 14 добу після внутрішньом'язового введення мезенхімальних стовбурових клітин у місці пошкодження відзначалися повністю сформовані м'язові волокна та міжм'язова сполучна тканина. У кролів після внутрішньом'язового введення алогенних мезенхімальних стовбурових клітин усі регенераторні процеси проходили активніше за такі у тварин інших дослідних груп і повністю завершувалися на 14 добу досліду. Практичною цінністю отриманих результатів є з'ясування закономірностей розвитку репаративних процесів в ушкодженій ділянці м'яза та визначення ефективності використання трансплантованих алогенних мезенхімальних стовбурових клітин у стимуляції відновлювальних процесів, що важливо враховувати в лікуванні м'язових травм із застосуванням регенеративної терапії

Ключові слова: клітинна інженерія; регенерація; двоголовий м'яз стегна; травма; скелетна мускулатура; гістологічні методи

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The effect of mesenchymal stem cells on platelet function in rats with experimental lung injury

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Abstract. Interstitial lung tissue diseases (pulmonary fibrosis) are accompanied by a decrease in thrombopoiesis. Stem cells are capable of differentiating into other cell types, making them a valuable material for veterinary cellular regenerative therapy. The purpose of the research – to explore changes in platelet counts in laboratory rats with experimental pulmonary fibrosis under the influence of stem cells. The research was performed on female *Wistar* rats, in which pulmonary fibrosis was modelled using a single transthoracic injection of bleomycin hydrochloride solution. Allogeneic mesenchymal stem cells were used to stimulate recovery processes in pathologically altered lung tissue, which were administered by different routes, and, for comparison, the conventional method of treatment. The presented results of the effect of transplanted allogeneic bone marrow mesenchymal stem cells indicate a significant change in the number and size of platelets in rats with experimental pulmonary fibrosis and an increase in the activity of regenerative processes in damaged tissues. In experimental animals, a significant increase in the number of platelets and their size was found after using allogeneic mesenchymal stem cells compared to similar data in control group animals. In addition, in the blood of animals of the experimental group, which were transplanted with mesenchymal stem cells transthoracically (directly into the lung tissue), there was a higher platelet activity than in animals with intravenous injection of mesenchymal stem cells. Platelet activation indicates an improvement in the regenerative capacity of damaged lung tissue under the influence of mesenchymal stem cells. Thus, transplanted mesenchymal stem cells stimulate platelet activity and regenerative processes in pathologically altered lung tissue in experimental fibrosis, which can be used as one of the effective methods of treating animals with this type of pathology

Keywords: allogeneic; pulmonary fibrosis; lung tissue; platelets; laboratory animals

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Introduction

Pulmonary fibrosis is a progressive chronic inflammatory lung disease with a complex pathogenesis. It is now known that wound healing mechanisms caused by repeated damage to the alveolar epithelium lead to progressive fibrosis (Pitchford *et al.,* 2019). Fibrosis is characterised by inflammatory cell infiltration, fibroblast proliferation and collagen deposition, which ultimately leads to irreversible lung function impairment (Yount *et al.,* 2016). Currently, there are no effective treatments for pulmonary fibrosis.

Mesenchymal stem cells are capable of differentiating into specialised cells of various types (more than 350) and exhibit immunomodulatory and anti-inflammatory effects. Their multipotency and migration capabilities are of interest to scientists and practitioners of human and veterinary medicine and biology, which has resulted in a large number of studies on their use for therapeutic purposes (Tzouvelekis *et al.,* 2018). In a research of bleomycin-induced pulmonary fibrosis, scientists found that using mesenchymal stem cells increased survival compared to conventional treatments (Reddy *et al.,* 2016). Under the influence of mesenchymal stem cells, there is a decrease in the activity of interleukins, tumour necrosis factor-α, and transforming growth factor-β, which decreases inflammation. In addition, there is a downregulation of matrix metalloproteinase with a decrease in collagen deposition and fibrosis (Koupenova *et al.,* 2018). Mesenchymal stem cells at the site of injury activate resident stem cells and their differentiation into local cell types (Morrison *et al.,* 2017).

Platelets and neutrophils play an important role in the progression of pneumonia (Zhan *et al.,* 2020). A series of studies have demonstrated that platelets are involved in the pathogenesis of many diseases (Levoux *et al.,* 2021). Platelet activation is triggered in the early stages after lung tissue damage, and capillary integrity is compromised when a cascade of coagulation reactions is activated. Activated platelets contribute to fibrosis through the release of active substances from their granules (Lai *et al.,* 2018; Łukasik *et al.*, 2018) or through interaction with immune and inflammatory cells (Lin *et al.,* 2017).

However, the potential role of platelets in pulmonary fibrosis has not yet been fully explored. Therefore, scientists have analysed the role of platelets in the onset of pulmonary fibrosis and changes in lung function caused by repeated exposure to low levels of bleomycin (Carrington *et al.,* 2021), as platelets accumulate and release mediators such as transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), which are implicated in the fibrosis process (Pitchford *et al.,* 2019). It is known that platelets are among the first cells to respond to injury and can undergo diapodosis into the extravascular space, where the release of profibrotic factors can contribute to the development of fibrosis (Eisinger *et al.,* 2018). It is now confirmed that the lungs are the main source of platelet production and their share, in particular, in mice is about 50% of the total production of these cells in the blood (Lefrançais *et al.,* 2017).

Currently, it has been established that platelets can promote wound healing and play an important role in haemostasis by aggregating at sites of vascular damage with the subsequent release of growth factors, which, according to some scientists, are involved in the remodelling of certain tissues (Petito *et al.,* 2018; Pitchford *et al.,* 2019). In addition, platelets are involved in the pathogenesis of several diseases with inflammation, primarily respiratory diseases: asthma, acute lung injury, obstructive pulmonary disease, and infectious diseases with predominant lung tissue damage (Petito *et al.,* 2018; Zaid *et al.,* 2020).

Thrombocytopenia is frequently manifested in lung pathology and is associated with impaired platelet haemostasis. The association between lung damage and thrombocytopenia was further explored *in vitro* in rats with lung damage and found that the number of platelets in their peripheral blood was significantly lower than in control animals. Therefore, the lungs may play an active role in regulating platelet counts, and lung damage results in a decrease in the number of circulating platelets (Petito *et al.,* 2018).

The purpose of the study – to establish changes in the number and size of platelets in the blood of laboratory rats of the *Wistar* line with experimental pulmonary fibrosis under the influence of transplanted allogeneic mesenchymal stem cells, depending on the routes of their administration. To obtain this purpose, the following tasks were solved: to clarify the specific features of the pathogenesis of fibrotic lung diseases; to determine the clinical manifestations of pulmonary fibrosis; to determine changes in the number of platelets and their size in the presence of pathology and with using mesenchymal stem cells.

Literature Review

Platelets – small, nucleated cell fragments that play an important role in haemostasis and thrombosis. Modern research has proven that platelets are immune cells and key immune modulators, capable of expressing various receptors and molecules that allow them to respond to pathogens and interact with other immune cells. These cells have been linked to the pathogenesis of several pathological conditions, such as asthma and idiopathic pulmonary fibrosis (Chebbo *et al.,* 2021).

Platelets are established as a result of the fragmentation of a megakaryocyte, a large polyploid cell. In the bone marrow, immature megakaryocytes undergo endomitosis with the subsequent development of polyploid nuclei. Polyploid megakaryocytes extend long cytoplasmic processes known as proplatelets and release them into sinusoidal blood vessels for further division and generate mature platelets. It has been established that a single megakaryocyte can produce from 100 to 1000 platelets (Machlus & Italiano, 2011; Semple *et al.,* 2011; Hou *et al.,* 2015).

Platelets have a lifespan of 7 to 10 days, and they are constantly being established to maintain a stable blood level and volume (Holinstat, 2017; Koupenova *et al.,* 2018; Łukasik *et al.,* 2018).

Researchers have confirmed that platelets can modulate the immune response by interacting with immune cells in various pathological and physiological processes, such as inflammation, tissue remodelling, and infection (Chebbo *et al.,* 2021).

Numerous observations have identified the potential role of platelets in the pathophysiology of pulmonary diseases (Zhan *et al.,* 2020). *In vivo* microscopy studies in laboratory animals, megakaryocytes have been identified as platelet-producing cells in the lung vasculature. Moreover, the lungs are responsible for almost 50% of the total platelet production (Lefrançais *et al.,* 2017). In addition, researchers have identified extravascular pulmonary megakaryocytes that are not involved in platelet production. RNA sequencing analysis has established that pulmonary megakaryocytes have an innate immunity phenotype compared to bone marrow megakaryocytes and may be involved in the regulation of the pulmonary immune response (Lefrançais *et al.,* 2017). These scientific results suggest that platelets may be involved in pulmonary immune haemostasis.

Researchers have identified that during the progression of pulmonary fibrosis, a variety of immune cells and inflammatory cells, including macrophages and neutrophils (Gregory *et al.,* 2015; Huang *et al.,* 2017) are involved in processes at different stages of the disease

(Keane *et al.,* 2005). Activated platelets contribute to neutrophil activation by releasing CD40 L, which binds to CD40 receptors on neutrophils (Storey & Sinha, 2016) or by secretion of P-selectin/CD62 P, which binds to the neutrophil-expressed glycoprotein P-selectin-1 (PSGL-1) ligand (Rahman *et al.,* 2014).

Platelets are involved both in coagulation processes under physiological conditions and contribute to the development of inflammation and wound healing (Stavenuiter *et al.,* 2013; Webster *et al.,* 2013). Platelet activation occurs in many types of diseases with inflammation and fibrosis (Pitchford *et al.,* 2019) and in the very early stages of lung damage. In this case, the inflammatory response may be secondary to platelet activation (Pitchford *et al.,* 2019).

Peraçoli *et al.* (2008) note that platelets can have both pro-inflammatory and anti-inflammatory effects. Alpha granules contain the anti-inflammatory factor transforming growth factor-β. They release interleukin-10, which inhibits the secretion of tumour necrosis factor-α by monocytes (Gudbrandsdottir *et al.,* 2013). It has been concluded that platelets inhibit the secretion of inflammatory mediators by macrophages through mechanisms involving cyclooxygenase during sterile or bacterial systemic inflammation (Xiang *et al.,* 2013).

Materials and Methods

The research was conducted in 2021. Experimental animals were kept and used for experimental studies according to the requirements of the current Law of Ukraine No. 3447-IV "On the Protection of Animals from Cruelty Treatment" (2006) and other bylaws, and Directives of the European Union 2010/63/EU (2010). In particular, the laboratory animals were kept in a separate room of the Povazhenko Department of Surgery and Pathophysiology in cages with 12-hour daylight hours at an air temperature of 20 to 23°C and with free access to water and food.

The object of the study was blood samples obtained from rats (with experimental pulmonary fibrosis), female *Wistar* rats, aged 4 months, with an average body weight of 277.0±4.26 g. During the preparatory period, lasting 45 days, pulmonary fibrosis was modelled in animals by a single instillation of 0.3 mL of bleomycin hydrochloride solution (Bleocin, Nippon Kayak Co., Ltd., Takasaki Plant, Japan) into the lungs at the rate of 1.0 mg/100 g of animal body weight in 0.3 mL of sterile physiological sodium chloride solution 0.9% at room temperature. The solution was injected once, transthoracically, straight into the lung tissue (Boiko *et al.,* 2013). The bleomycin solution was administered under light anaesthesia using telazol 100 mg/mL (Zoetis, Spain) at a dose of 30 mg/kg body weight, intramuscularly (Plumb, 2008) and in combination with Madison 0.1% (Brovapharma, Ukraine) at a dose of 0.25 mg/kg, intramuscularly (Plumb, 2008).

All animals with pronounced symptoms of pulmonary fibrosis on day 45 (baseline) were divided into 4 experimental groups of 20 animals each. Animals of the first experimental group were injected with mesenchymal stem cells transthoracically, directly into the lung tissue at a dose of 3 million/animal once, on the right side; animals of the second experimental group were injected with allogeneic mesenchymal stem cells intravenously at a similar dose. The animals of the third experimental group were treated with the conventional method of treatment – administration of dexamethasone solution 4 mg/mL (KRKA, Slovenia), at a dose of 0.08 mg/kg body weight, intramuscularly, for 3 weeks with an interval of 2 days with a gradual dose reduction, hyaluronidase solution 64 U (Lidaza-Biopharma, FZ "BIOPHARMA", Ukraine) at a dose of 0.85 U/kg, intramuscularly, for 3 weeks with an interval of 2 days (Boiko *et al.,* 2013); control group animals were injected with 0.3 mL of phosphate-buffered saline (Sigma,

USA) transthoracically, into the lung tissue directly, on the right side; the fifth experimental group was intact (healthy) animals.

Stem cells for use in the experiments were obtained from the bone marrow of young clinically healthy donor animals (rats) according to the methods developed by researchers of the Povazhenko Department of Surgery and Pathophysiology (Mazurkevych *et al.,* 2014).

The first day of mesenchymal stem cell injection was considered day zero (baseline). Blood samples for laboratory tests were taken randomly from 5 animals at baseline (before the experiment) and on days 7, 14, 30 and 45 of the experiment. For this purpose, the animals were euthanised by intraperitoneal injection (Shoyaib *et al.,* 2019) of a lethal dose of sodium thiopental (Thiopenate, Brovapharma, Ukraine) at a dose of 40 mg/kg body weight (Plumb, 2008). After the solution was injected into the abdominal cavity, euthanasia occurred due to the cessation of vital functions, namely breathing and heartbeat. Immediately after euthanasia, blood was immediately taken from the heart cavity for further haematological examination. The body of the euthanised animal was fixed in a dorsal position on a flat surface, perpendicular to the thorax. A puncture was performed near the sternum using a 2 mL syringe with a G 29 needle. The obtained blood samples were placed in special tubes with K3 EDTA (AQUISEL, Italy) to prevent the development of a blood clot and for further morphological examination on an automatic haematological analyser (Exigo BM800, USA) according to the manufacturer's instructions.

No animal deaths were recorded in the experimental groups during the experiment.

Statistical processing of the results was performed using the Microsoft Excel 2010 software package. The experimental data are presented as M±m (M – arithmetic mean; ± – standard error of the mean). The significance between the values of the control and experimental groups was determined by Student's t-test, and the values were considered significant at $P<0.05$.

Results and Discussion

Changes in the number of platelets in the blood of laboratory rats at different stages of the experiment have certain features depending on the method of application of stimulants for the restoration of damaged lung tissue in fibrosis and the period after the application of these methods (Table 1, Fig. 2).

Table 1. Platelet count in the blood of rats with experimental pulmonary fibrosis under the influence of mesenchymal stem cells and conventional treatment, 10^9/L, M±m, n=5

Stages of the experiment	Group of animals				
	1 experimental	2 experimental	3 experimental	4 (control)	5 experimental
Baseline state	650.20±35.93	650.20±35.93	650.20±35.93	650.20±35.93	668.30±28.30
7th day	895.80±5.96***	867.20±44.20***	420.00±46.36*	566.80±38.49	688.30±11.27
14th day	954.00±34.58***	940.00±27.56***	571.40±26.51	665.20±46.50	676.50±18.04
30th day	842.00±32.30***	893.00±6.21***	559.40±28.26	531.00±28.56	700.20±6.21
45th day	884.20±15.80***	879.60±20.44***	611.70±20.60	597.00±14.03	718.90±19.08

Note: **P<0.05; *P<0.01; ***P<0.001, significantly compared with control group animals*
Source: *author's development*

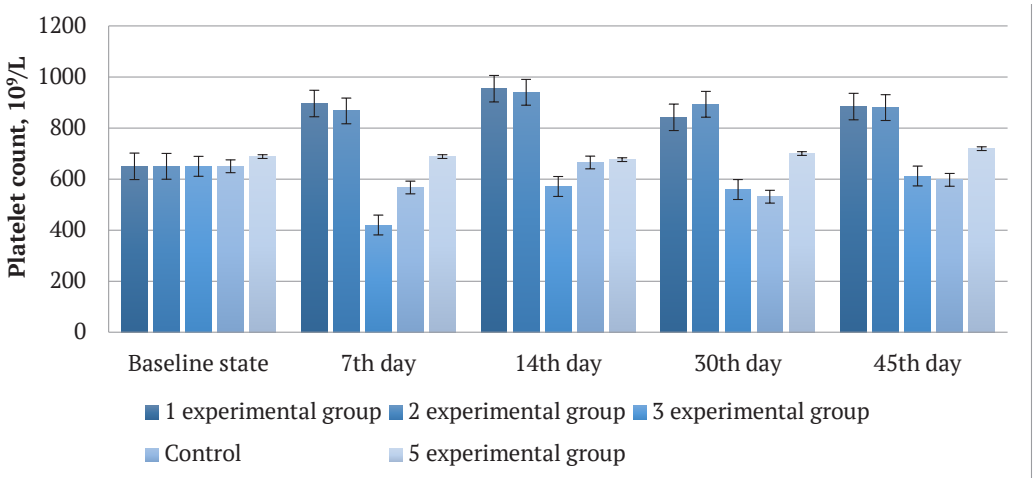

Figure 1. Dynamics of platelet count in the blood of rats with experimental pulmonary fibrosis under the influence of mesenchymal stem cells and conventional treatment

Thus, in the baseline state (before using the drugs), the platelet count in the blood of animals with experimental pulmonary fibrosis was lower than that of intact animals. Evidently, this is a consequence of the development of a pathological process in the lungs due to the modelling of experimental fibrosis in animals.

On day 7 of the experiment, after using allogeneic mesenchymal stem cells, a significant increase in the examined indicators was found in the blood of animals of groups 1 and 2 compared to those of the control group (group 4). In particular, a significant increase in the number of platelets by 37% ($P<0.001$) and 35% ($P<0.001$), respectively, was found in the blood of animals of groups 1 and 2 compared to the control group. A significant decrease in the number of platelets by 26% ($P<0.05$) was found in the blood of animals of group 3 compared to the baseline.

In the blood of animals of the control group, the number of platelets remained lower than in intact animals. The lowest number of platelets was recorded in the blood of animals of experimental group 3 (conventional treatment), and it was significantly lower than in the control group by 26% ($P<0.05$).

On the 14th day of the experiment, the number of platelets in the blood of animals of experimental groups 1 and 2 significantly increased by 30% ($P<0.001$) and 29% ($P<0.001$), respectively, compared to the control group. On the contrary, the number of platelets in the blood of animals of experimental group 3, which were treated with the conventional method, decreased by 14%.

On day 30 of the study, an increase in the examined indicators in the blood of animals of experimental groups 1 and 2 was found compared to the control group, but they were lower than the same indicators on day 14 of the experimental study. Thus, the number of platelets in the blood of animals of experimental group 1 significantly increased by 37% ($P<0.001$) and of experimental group 2 – by 40% ($P<0.001$) compared to the control group. A tendency to a slight increase in the number of platelets (by 5%) was observed in the blood of animals of experimental group 3.

On day 45 of the experimental study, an increase in quantitative indicators was noted in the groups of animals treated with allogeneic mesenchymal stem cells, in particular: in experimental group 1, the total number of platelets significantly increased by 32% ($P<0.001$), and in the blood of animals of experimental group 2, this indicator significantly increased by 30% ($P<0.001$) compared to the control group; in the blood of animals of experimental group 3, only a tendency to increase the number of these cells (by 2.4%) was recorded.

Thus, the results of the study indicate that transplanted allogeneic mesenchymal stem cells stimulate thrombocytosis in animals with pulmonary fibrosis. Therewith, the highest activity of platelet count increase was recorded in the first 14 days of the experiment when the regenerative processes in the damaged lungs were the highest. Subsequently, by day 45 of the experiment, the intensity of platelet growth decreased. The methods of conventional treatment (experimental group 3) do not contribute to a significant increase in the number of platelets, as evidenced by the comparison of the corresponding indicators in animals of experimental group 3 with those in control and intact rats.

Forty years ago, researchers were convinced that platelets were just "non-living" cell fragments that were only involved in the development of a platelet clot to stop bleeding (Leslie *et al.,* 2010; Garraud *et al.,* 2015). Only in the last decade has the number of discoveries about platelet biology and function increased, demonstrating (Andrade *et al.,* 2020; Levoux *et al.,* 2021) that these cells are among the most biotechnologically advanced. In addition, it has been reported in the literature that platelets enhance the wound-healing activity of mesenchymal stem cells, which is consistent with this research. However, the mechanisms by which they improve the therapeutic potential of stem cells remain unclear (Levoux *et al.,* 2021). There is evidence that after platelets are activated, they transfer respiratory-competent mitochondria to mesenchymal stem cells through dynamin- and clathrin-dependent endocytosis, which contributes to the therapeutic efficacy of these cells after engraftment and has been demonstrated in relevant tissue injury models in mice (Levoux *et al.,* 2021).

Therewith, in recent years, researchers have begun to explore platelet-rich plasma together with stem cells to stimulate the regenerative potential of mesenchymal stem cells (Andia *et al.,* 2018; Henschler *et al.,* 2019). Several studies have demonstrated that using platelet-rich plasma (PRP) increases the therapeutic efficacy of stem cells (Qian *et al.,* 2017; Mahmoudian-Sani *et al.,* 2018; Hersant *et al.,* 2019) and improves their survival after exposure to oxidative stress (Hersant *et al.,* 2019). For example, Qian *et al.* (2017) found that PRP (a natural product) isolated from whole blood is capable of producing numerous growth factors to regulate physiological activities. These factors have a stimulatory effect on the proliferation and differentiation of various stem cells in injury models.

Nowadays, Etulain (2018) has proven the stimulating role of growth factors released by activated platelets in wound healing. They operate as a result of their paracrine secretion, focusing on the participation of respiratory-competent mitochondria, both in membrane-encapsulated microparticles and as free organelles, during stem cell-based therapies (Marcoux *et al.,* 2017). Two-photon microscopy in live animals transfused with blood demonstrated that extracellular mitochondria interact with neutrophils *in vivo*, causing neutrophil adhesion to the endothelial wall (Marcoux *et al.,* 2017). In addition, these scientists noted that PRP treatment stimulates the proangiogenic potential of mesenchymal stem cells in mice *in vitro*, depending on the dose, through increased secretion of soluble factors such as VEGF and

SDF-1. Therewith, the therapeutic use of PRP in laboratory animals improves survival and activates the proliferation of *in vitro* cultured mesenchymal stem cells. These effects are accompanied by changes in energy metabolism in these cells, including the rate of oxygen consumption and production of adenosine triphosphoric acid in the mitochondria. However, the molecular mechanism and consequences of this phenomenon have not been fully understood. Only two studies have described that mitochondria released by activated platelets can be absorbed by platelets, which triggers an immune or regenerative response (Boudreau *et al.,* 2014; Zhao *et al.,* 2017).

During the experiment, the average volume of platelets changed according to the indicators of their quantitative composition (Table 2, Fig. 2).

Table 2. Changes in the mean platelet volume in the blood of rats with experimental pulmonary fibrosis and under the influence of mesenchymal stem cells and conventional treatment, fl, M±m, n=5

Stages of the experiment	Group of animals				
	1 experimental	2 experimental	3 experimental	4 (control)	5 experimental
Baseline state	5.36±0.05	5.36±0.05	5.36±0.05	5.36±0.05	6.06±0.10
7th day	7.18±0.23**	7.54±0.25***	5.26±0.05*	5.88±0.21	6.10±0.05
14th day	9.54±0.26***	9.52±0.28***	5.38±0.04	5.54±0.08	6.17±0.06
30th day	6.62±0.06***	6.18±0.29*	5.88±0.21*	5.38 0.04	6.10±0.12
45th day	6.34±0.18**	6.15±0.05***	6.02±0.06**	5.54±0.10	6.14±0.13

Note: **P<0.05; **P<0.01; ***P<0.001, significantly compared with control group animals*
Source: *author's development*

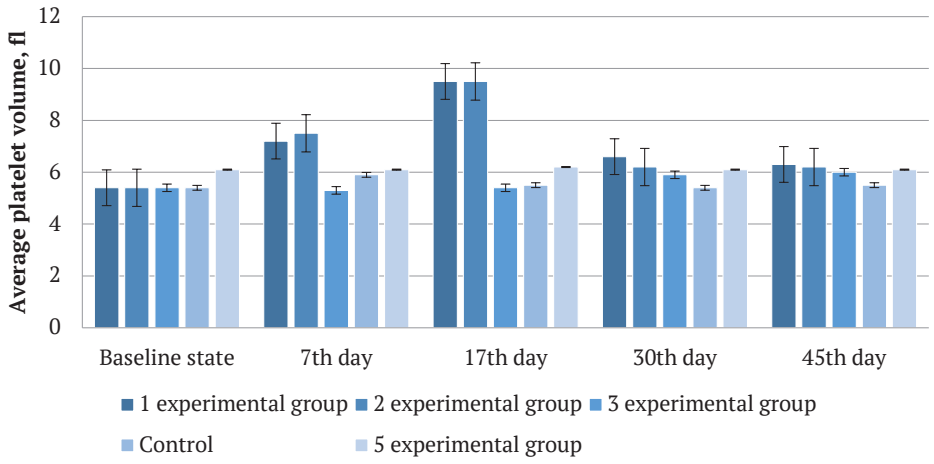

Figure 2. Changes in the average platelet volume in the blood of rats with experimental pulmonary fibrosis under the influence of MSCs and conventional treatment

Thus, on day 7 of the experiment, the average platelet volume in the blood of animals of experimental groups 1 and 2 significantly increased by 18% (P<0.01) and 22% (P<0.001), respectively, compared to animals of the control group. In animals of the experimental group 3, on the contrary, this index significantly decreased by 10% (P<0.05) compared to the control group and intact rats.

On day 14 of the experiment, the average platelet volume in the blood of rats of experimental groups 1 and 2 significantly increased by 42% (P<0.001) and 41.8% (P<0.001), respectively. In animals of experimental group 3, which were treated with the conventional method of treatment, the examined index decreased by 3% compared to the control group, but it was already higher than on day 7 of the study.

On day 30 of the experiment, the average platelet volume in the blood of animals of experimental group 1 significantly increased by 19% (P<0.001), and in rats of experimental group 2 – 13% (P<0.05). A significant increase in the average platelet volume by 8.5% (P<0.05) was observed in animals of experimental group 3. On day 45 of the experiment, the average platelet volume significantly increased in animals of all experimental groups: in experimental group 1 – by 13% (P<0.01), experimental group 2 – by 10% (P<0.001), and experimental group 3 – by 8% (P<0.01).

Thus, compared to intact animals, both the number of platelets and their average volume in animals of experimental groups 1 and 2 significantly increased in the first 14 days of the experiment after using mesenchymal stem cells. However, in animals of experimental group 3, which received conventional treatment, these indicators were lower, which is obviously due to the delay or absence of a stimulating effect on thrombocytosis and, accordingly, on the anti-inflammatory activity of platelets.

Thus, platelets are circulatory components derived from megakaryocytes present in the bone marrow that play an important role in physiological and pathophysiological processes such as thrombosis, haemostasis, inflammation and wound healing (Holinstat, 2017). Platelets contain cytokines or growth factors that initiate the regenerative process of ageing. They begin to be activated by biologically active growth factors in the target area through a paracrine effect. At the same time, platelets release growth factors that affect the activation of macrophages and stem cells at the site of damaged tissue. In addition, Du Toit *et al*. (2007) emphasise that tissue regeneration is facilitated by the proliferation and differentiation of stem cells. These nucleus-free cells contain secretory organelles, including alpha granules, containing various proteins and growth factors. As described by Gremmel *et al.* (2016) platelets contain functional mitochondria that provide energy for their metabolism.

Gomperts & Strieter (2007) note that in pathologically altered lungs, regenerative processes can be impaired by the depletion of specific chemical factors, which we observed in the control group. It is noted that the supplementation of these factors in combination with platelets can more effectively stimulate the regeneration of functional blood vessels and alveolar structures in pathologically altered lung tissue (Mammoto *et al.,* 2016).

Mammoto *et al.* (2016) demonstrated that platelets stimulate vascular and alveolar regeneration of the adult mouse lung via Ang1-LRP5-Tie2 signalling. These results may improve the efficiency of lung regeneration and lung organ engineering, as unregulated angiogenesis contributes to the pathogenesis of various chronic lung diseases.

Hersant *et al.* (2019) note that activated platelets potentiates several important properties of mesenchymal stem cells, including their proliferation, survival and angiogenic potential, which is why an increase in platelet count

was observed in animals treated with stem cells. Rodrigues *et al.* (2010) and Lai *et al.* (2018) confirmed that platelets exert their cytoprotective and mitogenic effects on mesenchymal stem cells through the production of mitogenic growth factors. Therewith, as noted by Gurtner *et al.* (2008) and Sadtler *et al.* (2016), platelet factors play an important role throughout the healing process of damaged tissues.

Since interstitial fibrotic diseases of the lung tissue disrupt the mechanisms of tissue repair, understanding how bone marrow mesenchymal stem cells regulate platelet activation may prove to be a strategy for regenerating damaged tissues.

Conclusions

As a result of using allogeneic bone marrow mesenchymal stem cells, their positive effect on thrombocytosis activity in an experimental study of lung injury leading to fibrotic changes in laboratory rats was established.

An increase in the number of platelets and their average volume was established in animals of experimental groups 1 and 2, which were transplanted with allogeneic mesenchymal stem cells. Thus, after transthoracic injection, there was an increase in platelets on days 7 ($P<0.001$), 14 ($P<0.001$), 30 ($P<0.001$) and 45 ($P<0.001$) compared to the control group. An increase in the average volume of erythrocytes was observed on days 7 ($P<0.01$), 14 ($P<0.001$), 30 ($P<0.001$) and 45 ($P<0.01$) of the experimental study. In the group of animals with intravenous stem tissue management, an increase in platelets was observed on days 14 ($P<0.001$), 30 ($P<0.001$) and 45 ($P<0.001$) and an increase in the average volume of erythrocytes on days 14 ($P<0.001$), 30 ($P<0.05$) and 45 ($P<0.001$).

During the application of the conventional method of treatment, an increase in the average platelet volume was found in the blood of animals on days 30 and 45, and in other studied parameters – on day 7. The transplanted mesenchymal stem cells stimulate the establishment of megakaryocytes, which are the precursors of platelets. As a result, the therapeutic efficacy of mesenchymal stem cells after their engraftment increases, since platelets can enhance the reparative properties of mesenchymal stem cells. Therefore, it is assumed that platelets may play an important role in the anti-fibrotic effect of stem cells.

The processes of platelet activation in fibrotic lung diseases and their molecular mechanisms under the influence of stem cells require further detailed research to determine the prospects of their use as an alternative to classical methods of regeneration of damaged tissues.

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Conflict of Interest

None.

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Вплив мезенхімальних стовбурових клітин на тромбоцитарну ланку в щурів за експериментального ушкодження легень

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Анотація. Інтерстиціальні захворювання легеневої тканини (фіброз легень) супроводжуються зниженням процесів тромбопоезу. Стовбурові клітини здатні до диференціювання в інші типи клітин, завдяки чому вони стають цінним матеріалом для ветеринарної клітинної регенеративної терапії. Мета роботи – дослідити зміни показників тромбоцитарної ланки у лабораторних щурів з експериментальним фіброзом легень за впливу стовбурових клітин. Дослідження проведені на щурах-самицях лінії *Wistar*, в яких моделювали фіброз легень із застосуванням однократного трансторакального введення розчину гідрохлориду блеоміцину. З метою стимуляції відновлювальних процесів у патологічно зміненій легеневій тканині використовували алогенні мезенхімальні стовбурові клітини, які вводили різними шляхами, та, для порівняння, – традиційний метод лікування. Наведені результати впливу трансплантованих алогенних мезенхімальних стовбурових клітин кісткового мозку свідчать про вірогідні зміни кількості та розміру тромбоцитів у щурів з експериментальним фіброзом легень, а також про підвищення активності регенеративних процесів в ушкоджених тканинах. У дослідних тварин встановлено вірогідне збільшення кількості тромбоцитів та їх розмірів після застосування алогенних мезенхімальних стовбурових клітин порівняно з аналогічними даними у тварин контрольної групи. Крім того, в крові тварин дослідної групи, яким трансплантували мезенхімальні стовбурові клітини трансторакально (безпосередньо в легеневу тканину), відмічали більшу активність тромбоцитів, ніж у тварин з внутрішньовенним уведенням мезенхімальних стовбурових клітин. Активація тромбоцитів вказує на покращення регенеративної здатності ушкоджених тканин легень за впливу мезенхімальних стовбурових клітин. Таким чином, трансплантовані мезенхімальні стовбурові клітини стимулюють активність тромбоцитів та регенеративні процеси в патологічно зміненій легеневій тканині за експериментального фіброзу, що може бути використано як один із ефективних методів лікування тварин за цього виду патології

Ключові слова: алогенні; легеневий фіброз; легенева тканина; тромбоцити; лабораторні тварини

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