

УКРАЇНСЬКИЙ ЧАСОПИС ВЕТЕРИНАРНИХ НАУК

Засновник:

Національний університет біоресурсів і природокористування України

Рік заснування: 2010

*Рекомендовано до друку та поширення
через мережу Інтернет Вченою радою*

*Національного університету біоресурсів і природокористування України
(протокол № 12 від 29 травня 2024 р.)*

**Рішення Національної ради України
з питань телебачення і радіомовлення № 1795,
протокол № 31 від 21.12.2023 р.
Ідентифікатор медіа - R30-02293.**

Журнал входить до переліку наукових фахових видань України

Категорія «Б». Галузь наук – Ветеринарні,
спеціальність – 211 «Ветеринарна медицина»,
212 «Ветеринарна гігієна, санітарія і експертиза»
(Наказ Міністерства освіти і науки України від 28 грудня 2019 р. № 1643)

**Журнал представлено у міжнародних наукометричних базах даних,
репозитаріях та пошукових системах:** Index Copernicus International,
Google Scholar, Index Copernicus, Фахові видання України, Національна бібліотека України
імені В. І. Вернадського, BASE, Directory of Open Access Journals (DOAJ), CAB Abstracts and
Global Health (CABI), Polska Bibliografia Naukowa (PBN), Ulrichsweb, Dimensions, University
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03041, вул. Героїв Оборони, 15, м. Київ, Україна
E-mail: info@veterinaryscience.com.ua
<https://veterinaryscience.com.ua/uk>

UKRAINIAN JOURNAL OF VETERINARY SCIENCES

Founder:

National University of Life and Environmental Sciences of Ukraine

Year of foundation: 2010

*Recommended for printing and distribution
via the Internet by the Academic Council
of National University of Life and Environmental Sciences of Ukraine
(Minutes No. 12 of May 29, 2024)*

**Decision of the National Council
of Television and Radio Broadcasting of Ukraine No. 1795,**
Minutes No. 31, dated 21.12.2023.
Media identifier - R30-02293.

The journal is included in the list of professional publications of Ukraine
Category “B”. Veterinary, specialty – 211 “Veterinary Medicine”, 212 – “Veterinary Hygiene,
Sanitation and Examination” (Order of the Ministry of Education and Science of Ukraine
of December 28, 2019, No. 1643)

**The journal is presented international scientometric databases, repositories
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2024. Volume 15, No. 2. P. 9-26

Received: 19.01.2024 Revised: 20.04.2024 Accepted: 29.05.2024

DOI: 10.31548/veterinary2.2024.09

UDC: 636.09:616.98

Epizootological and aetiopathogenetic aspects of mycobacterioses

Volodymyr Busol*

Doctor of Veterinary Sciences, Professor
National Scientific Center "Institute of Experimental and Clinical Veterinary Medicine"
61023, 83 Pushkinska Str., Kharkiv, Ukraine
<https://orcid.org/0000-0002-9618-0493>

Petro Boiko

Doctor of Veterinary Sciences, Associate Professor
Lesya Ukrainka Volyn National University
43025, 13 Voli Ave., Lutsk, Ukraine
<http://orcid.org/0000-0003-1418-1159>

Michał Bednarski

Doctor of Veterinary Medicine, PhD in Veterinary Sciences
Wrocław University of Environmental and Life Sciences
50-375, 25 C.K. Norwida Str., Wrocław, Poland
<https://orcid.org/0000-0003-0292-7929>

Viktor Shevchuk

PhD in Veterinary Sciences, Associate Professor
National University of Life and Environmental Sciences of Ukraine
03041, 15 Heroiv Oborony Str., Kyiv, Ukraine
<https://orcid.org/0000-0002-5937-3333>

Olha Panivska

Postgraduate Student
Lesya Ukrainka Volyn National University
43025, 13 Voli Ave., Lutsk, Ukraine
<http://orcid.org/0000-0002-1280-4458>

Abstract. Animal mycobacterioses, unlike those in humans, are not in the focus of veterinary science and practice for various reasons. This fact, as well as some others, including the growing incidence of human mycobacteriosis caused by non-tuberculous mycobacteria involved in the

Suggested Citation:

Busol, V., Boiko, P., Bednarski, M., Shevchuk, V., & Panivska, O. (2024). Epizootological and aetiopathogenetic aspects of mycobacterioses. *Ukrainian Journal of Veterinary Sciences*, 15(2), 9-26. doi: 10.31548/veterinary2.2024.09.

*Corresponding author



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sensitisation of productive animals, is an objective, albeit indirect, indication of the existing problem of mycobacteriosis in veterinary medicine. This study employed the comparative geographical and epizootological methods of analysis. The findings obtained from the publications of scientists from around the world helped to summarise and objectively assess the severity of the epizootic situation regarding mycobacteriosis in animals in the world and in Ukraine, to identify a range of features of the infectious and epizootic processes, and to establish the dominant aetiopathogenetic criteria for this infection in animal populations. Animal mycobacteriosis is the subject of detailed scientific research in most countries of the world, as this disease is widespread among productive and unproductive (domestic and wild) animals, as well as the fish fauna of sea and river waters. It was found that the most common causative agents of mycobacteriosis are nontuberculous mycobacteria such as *Mycobacterium avium complex*, *M. abscessus complex*, *M. kansasii*, and *M. fortuitum*, and some others. In Ukraine, animal mycobacteriosis is not given due attention, even though parallergetic reactions to tuberculin in cattle lead to complications in the diagnosis of tuberculosis infection. They cause considerable losses in dairy farming due to the culling and slaughter of highly productive cows. Identification of aetiopathogenetic patterns of paraallergic reactions to tuberculin in productive animals is a key to effective control and management of the epizootic process of animal mycobacteriosis and reduction of biohazards of human infection with non-tuberculous mycobacteria through food of animal origin

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

Introduction

Animal mycobacterioses, among other well-known problems of modern veterinary medicine, have become an objective issue in many countries. According to O.A. Zhurylo *et al.* (2020) and the World Health Organisation (2022), mycobacterioses of cattle – animals that are the main producers of milk and dairy products – are of the greatest epizootological and biomedical significance. The findings from L. Varela-Castro *et al.* (2022) also support this. The detection of nonspecific (paraallergic) reactions to tuberculin, primarily in cows from dairy farms that are free of tuberculosis, is a common phenomenon in many countries, including Ukraine. However, such para-allergic reactions to tuberculin in cattle are not usually recorded by specialists of state veterinary institutions, as they are deeply rooted in the opinion that non-tuberculous mycobacteria (NTMB) do

not cause an infectious process in animals, but only cause sensitisation of the animal body.

Based on this postulate, developed back in the 1950s, a misconception is being built that animal sensitisation is not the result of a latent infectious process caused by NTMB, but a permanent, harmless phenomenon that is not related to the infectious process. Thus, if there is no infectious process, then there can be no question of an epizootic process. Mycobacteriosis is an infectious disease caused by NTMB, which has its own aetiopathogenesis, inherent pathological and specific histomorphological changes, and is characterised by a predominantly asymptomatic, permanent form with sensitisation and chronic course. And as indicated by P.K. Boyko *et al.* (2021), the presence of an infectious process in one third of the dairy cows in the herd of a dairy farm is indisputable

evidence of the functioning of the epizootic process of mycobacteriosis in the herd.

The epidemiological monitoring of diseases caused by ATMB can become the basis for creating an information base for in-depth investigation of the epidemiology of mycobacterioses and the ecology of the causative agents of these infections to develop further concepts for the control and management of infectious and epizootic processes of mycobacterioses in animals. This, as pointed out by V.O. Busol *et al.* (2014), will allow to consider milk and dairy products not only as dietary foods, but also as a potential factor of NTMB transmission from AMB-infected cows to the public. Monitoring for the presence of NTMBs in raw milk may eventually become a mandatory requirement of the DSTU as one of the criteria for preventing biohazards to human health from milk and dairy products. Therefore, the purpose of this review was to analyse the literature on the specific features of the epizootic process and the dominant aetiopathogenetic criteria of mycobacteriosis in animal populations at the global level.

To fulfil this purpose, the comparative geographical and statistical methods, as well as the method of epizootological analysis, were used. Furthermore, in processing the scientific literature data, generally accepted research methods and techniques were employed, namely, scientific search, systematic approach, observation, generalisation, and description of published experimental data of other researchers, etc. The authors summarised and provided their own assessment of the main conclusions of the researchers of each of the scientific sources.

Environmental mycobacteria – potential causative agents of mycobacteriosis in animals

Mycobacteriosis is an infectious disease of humans and animals caused by representatives of a large group of NTMBs. V.N. Dahl *et al.* (2023) found an increasing role of NTMB in

the incidence of pneumonia in Denmark, while I. Pavlik *et al.* (2021) established the clinical manifestation of mycobacteriosis in fish, animals, and humans caused by *M. fortuitum*.

According to F. Jamal & M. Hammer (2022), in the early years, according to the introduced classification system for non-tuberculous mycobacteria, they were also called atypical mycobacteria (AMB). Presently, according to J.O. Falkinham (2013), they have been given another name – environmental mycobacteria. K.H. Schroder *et al.* (1992) report that ATMBs were identified shortly after they were found to cause human lung disease in the 1950s. According to Ž. Cvetnić *et al.* (2018), in 1954, A. Timple and E. Runyon proposed a classification of NTMBs, which is now called the Runyon classification. According to this classification, mycobacteria are divided into 4 main groups based on growth rate, colony morphology, and pigmentation ability. According to O.S. Shevchenko *et al.* (2019), the group of NTMBs most commonly encountered in the clinical practice of humane medicine doctors includes *M. avium-intracellulare complex (MAC-complex)*, *M. kansasii*, *M. fortuitum*, *M. abscessus*, *M. chelonae* and a range of others. They are often released from water, soil, and other environmental objects.

On the other hand, the unique structure of the mycobacterial cell wall, which contains many fatty and waxy compounds, substantially increases both the adaptive properties of mycobacteria to changing environmental conditions and their resistance to phagocytosis. This, according to D.T. Armstrong *et al.* (2022), provides mycobacteria with the ability to survive in the environment for a long time and is thus one of the determining factors in the considerable spread of mycobacteria among farm and wild animals.

Given the widespread distribution of ATMB in biotic and abiotic environmental objects, confirmation of their aetiopathogenic role in a particular case, detection of signs of sensitisation of the organism by them, and even

isolation and identification of the mycobacteria themselves requires the establishment of an infectious process in animals. Therefore, the available criteria for this may include the results of bacteriological and biological studies, which can be used to establish the presence of pathogenicity factors in the identified mycobacteria, or to detect pathological or pathomorphological changes in the organs and tissues of animals suspected of having the disease.

According to T.K. Marras *et al.* (2017), the American Association of Thoracic Surgeons has developed microbiological, clinical, and molecular genetic criteria to establish the aetiological role of NTMB in HIV-positive and HIV-negative patients. Unfortunately, such criteria have not yet been developed in veterinary medicine.

The results of the monitoring of scientific literature on the persistence of AMB in animal organisms suggest that a wide range of different animal species inhabiting the territories of countries on all continents are infected with them. The list of animals from which NTMBs have been isolated that have caused mycobacterioses includes productive agricultural, domestic, and wild animals, poultry, fish, amphibians, reptiles, and insects. The present review focused on those animal species and those types of NTMBs that can or do pose a biohazard to public health, both as evident and potentially pathogenic microorganisms.

Thus, according to K. Röltgen & G. Pluschke (2022), *M. ulcerans* infection, or Buruli ulcer, is a mycobacterial disease of humans in West and Central Africa, as well as Australia and Oceania, and is closely associated with tropical wetlands where *M. ulcerans* is found in water, marshes, fish, and insects. Experimental infections with *M. ulcerans* have been reproduced in lizards, amphibians, chicken embryos, opossums, armadillos, rats, mice, and cattle. Natural infection in animals has been observed only in Australia, specifically in captive koalas,

ring-necked possums, and alpacas. The lesions were clinically analogous to those observed in humans. From this review, it can be concluded that the parasitism of *M. ulcerans* on amphibians and warm-blooded animals, as well as humans, is optional, while the ecology of the pathogen itself indicates a saprophytic mode of life as the main one. That is, in the evolution of this AMB species, a gradual transition from saprophyticism to pronounced parasitism is observed, which suggests considering it a potential pathogen of a dangerous infectious disease of animals and humans.

Analysing the epidemiological features of mycobacterioses, O.A. Zhurylo *et al.* (2020) report that up to 80% of human mycobacterioses are caused by *M. avium complex*. They believe that the main reservoir of *M. avium* is open water. *M. avium* is transmitted from water to humans by aerosolisation as a result of the natural formation of aerosols above the water surface. Sick poultry are often the source of *M. avium*. Therefore, it can be argued that the infection caused by *M. avium complex* is sapronotic or zoonotic. This report raises the question: if open water is a reservoir of *M. avium*, then what is the source of this mycobacterium species from which it enters open water? Could water bodies be a habitat for mycobacteria? Scientific research on the ecology of *M. avium* may provide answers to these questions.

Infectious and epizootic processes in mycobacteriosis in Ukraine

Mycobacteriosis as a nosological unit of infectious pathology in veterinary medicine does not yet have the recognition and even definition that infections caused by mycobacteria of the tuberculosis complex (*M. tuberculosis*, *M. bovis*, *M. africanum*) have. The World Organisation for Animal Health (OIE) does not include animal mycobacterioses in epizootic monitoring. Therefore, it is impossible to give an objective

assessment of the spatial and temporal dynamics of the intensity of the epizootic process of mycobacteriosis in the world and in Ukraine specifically. Therefore, to fulfil this purpose, the materials of scientific publications related to this issue were used. The findings of these studies were obtained using different methods and did not always have the same purpose, which also bears the imprint of non-uniformity of the data being compared.

To assess the severity of the epizootic situation with mycobacteriosis in Ukraine objectively, it was necessary to first investigate the spatial and temporal features of the manifestation of mycobacteriosis in animals. The period of scientific observation included the years from 2000 to 2023. There are a range of reasons for the complexity of the task of assessing the epizootic situation in Ukraine. The objective reasons include a considerable reduction in the number of productive animals, the complete elimination of certain livestock industries (horse breeding, sheep breeding, rabbit breeding), and a considerable reduction in the period of keeping cows on dairy farms due to changes in milk production technology. The subjective reasons include the unsatisfactory level of diagnosis by veterinary practitioners and the lack of interest of specialists from state veterinary institutions in diagnosing new infectious pathologies, which adversely affected the reflection of the real state of the epizootic situation with mycobacteriosis, and hence the reduction in demand for the development of modern diagnostic methods and means of preventing infections caused by atypical mycobacteria (AMB).

B.M. Yarchuk *et al.* (2002) note that, as with any factor infection, the epizootic process of mycobacteriosis in animals is characterised by the interaction of three links – the source of the infectious agent, the mechanism of transmission of the pathogen, and the susceptible organism. This interaction takes place against

the backdrop of various environmental factors. Therewith, the latter in most cases play a decisive role in the manifestation of the infectious process of all factor infections, including mycobacteriosis. There is currently no consensus on the source of the causative agents of animal mycobacteriosis. However, P.K. Boyko *et al.* (2021) suggest that animals infected with AMB, including those that respond positively to AMA, are the main source of the infectious agent, because mycobacteria in their bodies are not just indifferent microorganisms, but also cause an active, albeit clinically latent, infectious process. Once in the body of animals, AMBs increase their pathogenicity and, when excreted into the environment in faeces, milk, or other secretions, infect other animals, which we observed in the cows of the dairy farm we studied.

However, considering that the problem of mycobacteriosis in animals is at the initial stage of its development, we believe that the first link of the epizootic chain, namely the characteristics of the source of the infection agent, has been understudied. V.M. Gorzheev *et al.* (2004) emphasise that the investigation of the ecology of different mycobacterial species in animals and in environmental objects allows determining the degree of activity of the pathogen in the manifestation of the epizootic process. Such a theoretical substantiation can be used for a comprehensive study of the epizootic process in both tuberculosis and other mycobacterioses.

L. Heifets (2004) believes that little attention is paid to the study of microbiological characteristics of AMB. This is explained by a range of reasons. As reported by L.M. Feazel *et al.* (2009), mycobacterioses do not have a tuberculosis-like clinical course, and therefore are difficult to detect by clinical diagnostic methods. They often form a mix of infections with tuberculosis, which is why diagnosticians overlook them. However, possessing a wide range

of resistance, AMBs can survive in the environment for a long time and infect susceptible animals for a considerable period of time, causing sensitisation or infection, which is determined by the virulence of mycobacteria.

According to E.M. Lipner *et al.* (2017), the source of pathogens in animal mycobacterioses is sick animals and bacterial carriers, as well as various environmental objects (feed, water, soil, manure), where mycobacteria can not only survive but also multiply, increasing their population, but also have certain ways of entering susceptible organisms, which from the standpoint of epizootology is the basis for classifying them as saprophytic. However, considering the genetically determined pathogenicity factors (cord factor; specific feature of the cell wall structure, which provides resistance to phagocytosis; ability to form L-forms; availability of mechanisms to overcome and inactivate adverse environmental factors, which puts them in second place after spore-forming microorganisms in the bacterial domain), R.A. Mercaldo *et al.* (2023) argue that a considerable number of *Mycobacterium* species evolve towards complete pathogens such as *M. avium*, *M. intracellulare*, *M. kansasii*, *M. fortuitum*, *M. abscessus*, *M. vaccae*, etc.

According to V.M. Gorzheev (2005) and A.I. Zavgorodnyi (2014), in Ukraine, during routine allergic studies, 200–280 farms annually detect reactive animals in which no lesions inherent in tuberculosis are found in organs and tissues during diagnostic slaughter. Bacteriological tests of biomaterial from such animals do not isolate the tuberculosis pathogen. However, non-tuberculous mycobacteria (NTMB) are isolated from the lymph nodes and submucosal layer of the cranial colon, including *M. fortuitum*, *M. scrofulaceum*, *M. xenopi*, etc. The studies of such Ukrainian scientists as V.M. Gorzheev (2005), G. Dyachenko *et al.* (2006), B. T. Stegnyy *et al.* (2014) proved that

over the past 30 years, the isolation of AMB, specifically *M. smegmatis*, *M. fortuitum*, *M. phlei*, *M. vaccae*, etc. has increased significantly (up to 86.2% of the total number of all isolated and identified mycobacteria) from tuberculin-reactive cattle.

The isolation of AMB adversely affects diagnosis and health promotion activities, especially in the case of recurrent tuberculosis. G. Dyachenko *et al.* (2006) note that the use of a comprehensive method of tuberculosis testing allows differentiating the nature of reactions to tuberculin in cattle during two cycles of diagnostic tests. Farms in which cattle are sensitised by AMB should be considered TB-free, as atypical mycobacteria in healthy animals do not cause the development of the TB process.

However, the question of how to deal with animals that tested positive for the atypical mycobacterial allergen (AMA) is still open. According to B.T. Stegnyy *et al.* (2014), these animals, as well as those that have shown par-allergic reactions to tuberculin, are considered free of TB and can stay in the herd without any restrictions. In this regard, the question stays as to the mechanism of sensitisation – whether it is a physiological or pathological reaction of the immune system in response to permanent or permanent ingestion of AMB into the body of animals or the result of an infectious process caused by AMB. The answers to this, as well as to many other related questions, can be obtained in a detailed investigation of the ways of infection of animals with AMB, their interaction at the cellular, tissue, organ, and organismal levels of infected animals.

A.I. Zavhorodniy & O.V. Kotlyar (2014) found that among cattle in TB-free farms in Ukraine, reactions to tuberculin (purified protein derivative) for mammals were caused by 13 species of atypical mycobacteria, including *M. gordone*, *M. scrofulaceum*, *M. avium*, *M. terae*, *M. xenopi*, *M. peregrinum*, *M. fortuitum*,

M. smegmatis, *M. vaccae*, *M. phlei*, *M. flavescens*, *M. diernhoferi*, *M. thermophiles*. The researchers proved that some isolated species of atypical mycobacteria caused in guinea pigs an increased delayed-type sensitivity to mammalian tuberculin (PPD) but did not cause pathological and anatomical changes inherent in tuberculosis.

However, the scientists do not mention the presence of histomorphological changes in the experimental animals. According to V.O. Busol et al. (2014), histological studies and bacteriological examination of regional lymph nodes in infected guinea pigs for the presence of AMB would provide a broader view of the mechanism of sensitisation, and therefore the nature of paraallergic reactions in both experimental animals and animals from which these mycobacteria were isolated. The data obtained by the researchers suggest that a considerable number of AMBs circulate among the cattle population in Ukraine, which complicate allergic diagnosis of tuberculosis in herds of productive animals and pose a threat of human infection through food of animal origin.

This review suggests that none of the Ukrainian scientists has addressed the underlying mechanisms of AMB-induced sensitisation in animals. It is unclear at what stage of interaction between a microorganism and a macroorganism sensitisation occurs, whether it is a result of a latent infectious process caused by AMB, or whether infection with AMB is a purely mechanical process of their penetration into the body with the subsequent activation of a delayed hypersensitivity reaction, which is detected by allergic testing. In the end, the mechanisms of extinction of this process are still unexplored, because paraallergic reactions to tuberculin are permanent, i.e., they can disappear after a certain period of time and then reappear.

The detection of nonspecific (paraallergic) reactions to tuberculin, primarily in cows from dairy farms free of tuberculosis, is a common

phenomenon in many countries. Thus, according to G. Dyachenko et al. (2006), A.I. Zavorodniy et al. (2018), in the United States, 10-15% of animals with paraallergic reactions to tuberculin are detected in routine allergic tests of cattle for tuberculosis, in France – 15-30%, in Latvia – 35.8%, in Lithuania – 81.8-98.3%, in Estonia – 92-95.1%.

These facts are indirect, but objective evidence that AMB circulate in cattle herds around the world, which are the main factors in the occurrence of non-specific reactions to tuberculin. On the other hand, it can be argued that the problem of infection of productive animals with AMB is relevant not only from a scientific standpoint, specifically, a deeper investigation of the aetiopathogenesis, but also from a practical standpoint – how to reduce “spontaneous” outbreaks of paraallergic reactions in a particular herd. The question persists whether these reactions are based on sensitisation of the body as a physiological reaction of the immune system in response to the ingestion of AMB, or sensitisation of the body as a result of an infectious process caused by these bacteria.

According to A.I. Zavorodniy et al. (2018), as of 1 January 2017, there were no more TB-affected locations in Ukraine. However, according to the World Organisation for Animal Health (OIE), Ukraine is currently not in a good situation regarding animal tuberculosis. This is evidenced by allergic reactions of cattle to tuberculin, which are constantly detected in many farms in Ukraine. Reports by V.Yu. Kassich et al. (2010), A.I. Zavorodniy et al. (2018) on the epizootic situation of tuberculosis and other mycobacterioses of cattle in Ukraine at the beginning of the 21st century indicates that the average annual number of dairy farms where reactive animals to tuberculin and allergen of atypical mycobacteria are detected is about 250 ± 30 , (1.47%) of all medium and large dairy farms in the country. The absence of a unified

reporting system of the State Service of Ukraine for Food Safety and Consumer Protection on existing cases of mycobacteriosis in animals does not allow for an objective characterisation of the spatial and temporal dynamics of the epizootic process of mycobacteriosis and the identification of epizootic features of this infection. Therefore, for the above reasons, it is not possible to show the distribution of mycobacteriosis foci by regions of Ukraine, and therefore to establish the territorial confinement of mycobacterial infections in each region.

Features of the manifestation of infectious and epizootic processes of mycobacteriosis of animals in countries around the world

To identify trends in the development of the epizootic process of mycobacteriosis on different continents, the study first analysed scientific publications related to the isolation and identification of AMB from productive animals, and more concretely from cattle in different countries of the world. The next step was to analyse the epidemiological situation regarding mycobacteriosis in other animal species, including amphibians, fish, and poultry.

A group of Brazilian scientists S.A. Bolaños *et al.* (2018) in 08.2014-08.2015 tested 4,766 cows with an intradermal simultaneous tuberculin test in 15 districts of the state of Paraná (Brazil) using PPD-tuberculin and an allergen from *M. avium*. 142 (2.98%) animals were positive. Most often (71%), cows aged 5-10 years were positive. 14 strains of mycobacteria (8.4%) were isolated from 12 cows, including *M. engbaekii* (n = 5), *M. nonchromogenic* (n = 3), *M. arupense* (n = 4), and *M. heraklionense* (n = 2). Despite the absence of *M. tuberculosis* complex mycobacteria in the milk samples, the results highlight the risk of transmission of pathogens from cattle to humans through milk, as some mycobacterial species identified in milk have

been identified as aetiological agents in pulmonary and extrapulmonary diseases in immunocompromised individuals.

In Brazil, where there are more than 220 million heads of cattle, the study of the ecology of mycobacterial pathogens is receiving due attention. Thus, R.B. Pardo *et al.* (2001) identified *Mycobacterium* spp. most frequently (78.95%) out of 780 raw milk samples from 52 cows with a positive simultaneous sample (Stormont test), followed by *M. fortuitum* (10.52%), *M. bovis* and *M. avium* (5.26% each). This is evidenced by scientific publications by C.Q. Leite *et al.* (2003), S.A. Sgarioni *et al.* (2014), which, among other things, even reported the isolation of NTMBs from pasteurised milk samples. Considering that Brazil is the largest country in Latin America, it can be argued that the prevalence of mycobacteriosis among productive animals, especially cattle, is high in other countries of the continent. This requires further research to develop effective measures to prevent mycobacteriosis in herds of productive animals.

NTMB in cow's milk was detected in Asian countries, specifically, M.F. Qamar *et al.* (2013) in Pakistan; F. Aydin *et al.* (2012) in Turkey, and R.R. Kazwala *et al.* (1998) in the African continent – Tanzania. According to C. Chang *et al.* (2002), NTMBs are widespread on dairy farms, being isolated from manure, feed, plants, and water. NTMB infection in cattle occurs nutritionally through the consumption of contaminated water and feed, and aerogenously. However, few studies have been devoted to the elimination of mycobacteria from the body of cows, especially those with positive tuberculin test results. In this case, the reports of G.S. Pandey *et al.* (2013), I. Ben Kahla *et al.* (2011) on the detection of *M. bovis* in milk samples from cows that reacted positively to tuberculin are of great interest. M.M. Franco *et al.* (2013) found that the majority of mycobacteria are eliminated by the intramammary route.

Thus, AMBs are widespread on the territory of dairy farms in many countries on different continents of the world – they are isolated both from abiotic environmental objects and from the body of cows. Therewith, they are identified in the milk of both clinically healthy cows and in the milk of cows that react positively to tuberculin and AMA, which suggests that they are not just transient in the body of cows, but actively multiply in it with all possible manifestations of this form of interaction, which is inherent in a clinically latent or chronic course of the infectious process, and not just sensitisation.

According to scientific publications, NTMBs are widespread among both domestic and wild-life animals. Thus, a group of Polish scientists Ł. Radulski *et al.* (2022) found that mycobacteriosis in animals is often caused by *M. avium* ssp. *hominissuis*. Many species of animals are susceptible to infection with this bacterium, even those in zoos. Therefore, another group of Polish scientists, M. Bruczyńska *et al.* (2023), investigated zoo animals for paratuberculosis (PTB). Out of 131 faecal samples collected from different animal species of eight zoos in Poland, 7 isolates of mycobacteria were bacteriologically isolated, including 5 – *M. avium*, 1 – *M. fortuitum*, and one unidentified species of *Mycobacterium*. PCR confirmed the presence of PTB in faeces from 9 animals. These data also suggest that the paratuberculosis pathogen circulates in animal populations in Polish zoos.

The Australian team of scientists C.R.O'Brien *et al.* (2014) investigated the spread and manifestation of the infectious process caused by *M. ulcerans* among wildlife (common, ringed, mountain, and brushtail possums). The mode of transmission and reservoirs of the bacterium in the environment is still unknown. Real-time PCR studies have detected *M. ulcerans* DNA in a variety of environmental samples, including faeces from local opossums with and without clinical signs of infection. In 27 clinical cases

of *M. ulcerans* infection in adult opossums, single or multiple ulcerative skin lesions were observed, mainly in the head, limbs, and tail, while in 4 cases, pathological examination revealed systemic disease. The natural course of the disease is still unknown, but some easily affected possums (common brush-tailed and mountain possums) can spontaneously overcome the infection, while in other animals (common and ringed possums) the infection can be potentially fatal. The authors believe that further work is needed to determine whether *M. ulcerans* infection poses a potential threat to opossum populations and whether these animals can act as reservoirs of the pathogen.

A group of Italian scientists A. Vetere *et al.* (2022) described a fatal case of systemic mycobacteriosis caused by *M. goodii* in a Kenyan sand boa (*Eryx colubrinus loveridgei*) captured in the wild. Histological examination of the whole organs confirmed systemic mycobacteriosis. *M. goodii* was identified by real-time PCR. South American scientists D.H. Morais *et al.* (2021) pay great attention to infectious diseases of wild animals, which are a potential source of human infectious agents. A total of 1,306 amphibian specimens belonging to 6 families, 12 genera, and 21 species were subjected to a comprehensive study. Of these, 17 samples (0.76%) showed petrified nodules in the intestinal cavity, kidneys, liver, lungs, intestines, and pancreas. The nodules were examined histologically and by PCR. As a result, *M. gordonae* was identified. This is the first report of *M. gordonae* infection in these amphibian species in the wild, while histopathology confirmed a typical mycobacterial lesion in amphibians in northeastern Brazil.

As the results of studies conducted by scientists from different countries suggest, mycobacteriosis is widespread in wild populations of both warm- and cold-blooded animals. Therewith, clinical manifestations of this infection were observed in animals in all countries where

comprehensive (clinical, pathological, bacteriological, histomorphological, and molecular genetic) studies of biomaterials were conducted. The most commonly identified aetiological agents include *M. ulcerans*, *M. gordonae*, *M. avium*, *M. fortuitum*, and many unidentified species. This suggests that animal mycobacteriosis is a widespread phenomenon in the wild fauna and requires a deeper and broader study of the specific features of both infectious and epizootic processes, as well as the establishment of the species composition of pathogens and their ecology. The findings obtained will be of both general biological and health and veterinary significance, as the data will be used to develop an effective system for controlling epizootic and epidemic processes of mycobacteriosis.

The monitoring of scientific studies on avian mycobacteriosis has shown that this subject has received considerable attention from scientists from around the world. Thus, a group of scientists from European universities R.K. Hoop *et al.* (1993) investigated six cases of mycobacteriosis caused by *M. genavense* in three budgerigars (*Melopsittacus undulatus*), one orange-winged amazon (*Amazon amazonica*), one blue-and-white flycatcher (*Cyanoptila cyanomelana*), and one Sunda zebra finch (*Taeniopygia guttata*). Pathological changes caused by NTMB included muscle wasting (5 cases), hepatomegaly (4 cases), and thickening of the small intestine wall (4 cases), as well as granulomas in the lungs and subcutaneous tissue (1 case each). Acid-fast bacilli were found in the liver of all 6 birds. *M. genavense* was isolated only on acidic BACTEC medium, while the pathogen was not isolated on egg medium. These findings suggest the significant role of the environment as a reservoir for *M. genavense*.

German scientists V. Schmidt *et al.* (2022) investigated mycobacteriosis of domestic and some wild birds and the factors contributing to their occurrence. A total of 50 birds diagnosed

with mycobacteriosis based on pathological changes were examined. It was found that the causative agents of mycobacteriosis in birds were *M. avium subsp. avium* (identified in 20 out of 50 birds), *M. genavense* (in 16 out of 50), and *M. avium subsp. hominissuis* (in 3 out of 50). Co-infections (psittacosis, virions, bacteriosis, mycosis, and endoparasitosis) had no effect on the manifestation of mycobacteriosis. Pathological changes of mycobacterial origin were most often (95%) detected in *M. avium* infection, while in *M. genavense* infection – 66%. Pathological changes were detected in the small intestine in 71% of the birds studied and in the lungs in 65%, indicating oral and pulmonary ingestion of mycobacteria. The new strains of *M. avium subsp. hominissuis* that were isolated and identified from birds are of epidemiological significance in terms of their zoonotic role, as this species is of increasing public health concern.

In the 1990s, a group of Czech scientists K. Hejlíček & F. Treml (1994) investigated the occurrence and spread of mycobacteriosis among pigeons, their susceptibility to infection and epizootological significance as a source of *M. avium*. A total of 470 pigeons from seven settlements with different epizootic situations regarding avian tuberculosis were studied. Tuberculosis lesions were detected in 2 birds (0.43%), and mycobacteria were isolated from 5 birds (1.06%). Four out of five isolated strains of mycobacteria were pathogenic to poultry. High resistance of pigeons to *M. avium* was experimentally proved.

A group of scientists from the University of Panama, C. Rengifo-Herrera *et al.* (2019), reported mycobacteriosis in a harpy eagle that showed extreme emaciation with dyspnoea, abnormal changes in the spleen, liver, and air sacs. The biochemical and haematological changes associated with the infection were not initially suspected to be mycobacteriosis. The diagnosis was confirmed by PCR analysis, which

revealed the presence of the *M. avium* complex in the tissue samples. A group of scientists from the United States, G. Pawsat *et al.* (2023), investigated a unique case of mycobacteriosis in a 32-year-old male salmon crested cockatoo (*Cacatua moluccensis*), which was histologically diagnosed with bilateral ulnar lymphoma. *M. genavense* was identified by PCR. This is an unusual case of simultaneous manifestation of two major diseases – mycobacteriosis and severe degenerative changes in the joints, which could lead to lymphoma.

The analysis of the above publications suggests that infection of birds with NTMB is quite common, and it can be associated with other pathologies and co-infections, which often leads to misdiagnosis. The pH of the medium in which we want to isolate mycobacteria from environmental objects is of particular importance. Clearly, this fact should be borne in mind not only in the microbiological laboratory when isolating mycobacteria from biomaterials and abiotic objects, but also when investigating the specific features of their ecology. Mycobacteriosis in fish is a well-known problem in ornamental fish farming worldwide, as well as in food fish species. Less is known about the prevalence of mycobacteriosis in wild fish species. Norwegian scientists N. Sandlund *et al.* (2023) described mycobacteriosis in Atlantic mackerel (*Scomber scombrus*) in the form of granulomatous kidney disease. *M. lentiflavum*, *M. stomatopiae*, and *M. montefiorensis* were isolated from 6 samples of mackerel caught in the North Sea.

The freshwater ornamental fish trade is the main source of livelihood for many producers in Trinidad and Tobago. The Aquatic Animal Health Unit of the School of Veterinary Medicine, University of the West Indies, has shown that fish mycobacteriosis is a quite common infection in local ornamental fish. This is based on the findings of studies of 122 fish samples by P. Savage *et al.* (2022). *Mycobacterium sp.* was

identified by real-time PCR. The prevalence of infection caused by *Mycobacterium sp.* was $61 \pm 7\%$ (74/122), with positive samples obtained from 54.2% (13/24) of the surveyed farms. Siamese fighting fish (*Betta splendens*) play a significant role in the global aquarium fish trade, but their susceptibility to mycobacteriosis could threaten the long-term sustainability of the industry. Furthermore, N.D. Hung *et al.* (2023) believe that the emergence of highly pathogenic and multidrug-resistant NTMB strains such as *M. chelonae*, *M. cosmeticum*, *M. farcinogenes*, *M. mucogenicum*, and *M. senegalense* isolated from *Betta splendens* fish has raised concerns both in terms of negative economic impact and public health risks.

Scientists from the University of Baghdad, F. Hussein *et al.* (2016), investigated the significance and role of *M. marinum* in human and animal infection. *M. marinum* is a species that causes mycobacteriosis in all species of fish (freshwater, brackish, and saltwater), as well as in humans and some animal species. In fish, transmission can occur through the consumption of contaminated food, cannibalism of infected fish or aquatic detritus, or through injury, skin abrasions, or external parasites. The infection in humans mostly occurs in people who keep aquarium fish at home. The infection can be an occupational disease for workers – fish processors or pet shops. Damage to the skin serves as an entry gate for the microorganism during contact with infected fish, during cleaning or maintenance of aquariums, after fin injuries or fish bites. Less commonly, infection can occur through contact with natural water sources while fishing, boating, or swimming. Contact with marine animals and reptiles plays a role in the transmission of the pathogen.

Spanish scientists, E. Montero *et al.* (2022), described in detail mycobacteriosis in syngnathids that were susceptible to NTMB infection. Clinical manifestations of the disease

ranged from sudden death to non-specific signs (anorexia, depressed general condition, weight loss, severe shortness of breath with increased respiratory rate). Visible ulcers were found on the tail and small white nodules in the liver and inside the eye. The gills, liver, intestines, and mesentery were most often affected. *M. fortuitum*, *M. chelonae*, *M. marinum*, and *Mycobacterium* spp. were identified by real-time PCR. R.S.M. Chong (2022) investigated cases of mycobacteriosis in fish. The first identified pathogen of fish mycobacteriosis was *M. piscium*. Over 200 species of fish found in waters ranging from the cold subarctic to tropical regions have been found to be infected with mycobacteria. Many species of *Mycobacterium* have been identified in fish that do not show clinical signs of disease. However, the most common causative agents of fish mycobacteriosis are *M. marinum*, *M. fortuitum*, and *M. chelonae*. Mycobacteriosis in fish is chronic and is characterised by exhaustion or poor body condition, loss of scales, skin ulcers, reproductive disorders and, rarely, deep haemorrhagic skin lesions. However, the researcher believes that the key takeaway is that fish mycobacteriosis is a zoonotic disease of people who come into contact with affected fish and the water bodies where they are found.

Summarising the above, further investigation of the spatial and temporal parameters of the spread of animal mycobacteriosis in Ukraine and other countries may reveal new patterns of manifestation of the epizootic process of animal mycobacteriosis and its driving forces, which will allow developing effective ways to control this infection not only in populations of different animal species, but will also guarantee food safety and, consequently, human health.

Conclusions

The epidemiological analysis of the spatial and temporal intensity of the epizootic situation regarding mycobacteriosis of animals caused by

AMB in Ukraine and some countries of different continents of the world for 2000-2023 revealed that mycobacterial infections are constantly occurring and are widespread both geographically and in terms of species. In Ukraine, mycobacteriosis was most often recorded in cattle, where it occurs in a latent asymptomatic form and is detected by a simultaneous test in the allergic diagnosis of tuberculosis using PPD-tuberculin and AMA, with additional postmortem bacteriological and histomorphological examination of biomaterial from reactive animals. Every year in Ukraine, 250 ± 30 dairy farms are registered, where AMA-reactive animals are detected, as well as individuals with parallergetic reactions to PPD-tuberculin, which indicates the circulation of AMB in Ukrainian dairy cow herds. *M. smegmatis*, *M. scrofulaceum*, *M. fortuitum*, *M. avium*, *M. phlei*, *M. vaccae* and a range of others, including *M. gordone*, *M. terrae*, *M. xenopi*, *M. peregrinum*, *M. flavescens*, *M. diernhoferi*, *M. thamnophaeos*, were identified as potential causative agents of mycobacteriosis in Ukraine.

On the territory of dairy farms in many countries on different continents of the world, AMBs are widespread, as they are isolated both from abiotic environmental objects and from the body of cows. Therewith, atypical mycobacteria are identified in the milk of both clinically healthy cows and in the milk of cows that react positively to AMA and tuberculin, which suggests that they are not merely transient in the animal body, but also actively multiply in it with all possible manifestations of this form of interaction, which is inherent in the latent course of the infectious process, not just sensitisation.

Mycobacteriosis is widespread in wild populations of both warm- and cold-blooded animals, as well as poultry and fish. Therewith, clinical manifestations of this infection were observed in all countries where comprehensive (clinical, pathological, bacteriological, histomorphological, and molecular genetic) studies

of biomaterials were conducted. The most commonly identified mycobacterial species include *M. ulcerans*, *M. gordonae*, *M. avium*, *M. fortuitum* in animals; *M. avium subsp. Avium*, *M. avium subsp. Hominissuis*, *M. genavense* in poultry; *M. marinum*, *M. fortuitum*, and *M. chelonae* in fish, and many unidentified mycobacterial species. This suggests that mycobacteriosis is a widespread phenomenon in the wild fauna and requires a deeper and broader study of the specific features of both infectious and epizootic processes, as well as the establishment of the species composition of pathogens and their ecology.

The prospect of further research will be to investigate the impact of NTMBs circulating on the territory of a particular dairy farm on the organism of productive animals to establish the ways of infecting animals, pathogenesis, and mechanism of excretion from the body of animals as potential pathogens of human mycobacteriosis.

Acknowledgements

None.

Conflict of Interest

None.

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Епізоотологічні та етіопатогенетичні аспекти мікобактеріозів

Володимир Бусол

Доктор ветеринарних наук, професор
Національний науковий центр «Інститут експериментальної і
клінічної ветеринарної медицини»
61023, вул. Пушкінська 83, м. Харків, Україна
<https://orcid.org/0000-0002-9618-0493>

Петро Бойко

Доктор ветеринарних наук, доцент
Волинський національний університет імені Лесі Українки
43025, просп. Волі, 13, м. Луцьк, Україна
<http://orcid.org/0000-0003-1418-1159>

Міхал Беднарські

Доктор філософії, кандидат ветеринарних наук
Вроцлавський університет наук про навколишнє середовище та життя
50-375, вул. Норвіда, 25, м. Вроцлав, Польща
<https://orcid.org/0000-0003-0292-7929>

Віктор Шевчук

Кандидат ветеринарних наук, доцент
Національний університет біоресурсів і природокористування України
03041, вул. Героїв Оборони, 15, м. Київ, Україна
<https://orcid.org/0000-0002-5937-3333>

Ольга Панівська

Аспірант
Волинський національний університет імені Лесі Українки
43025, просп. Волі, 13, м. Луцьк, Україна
<http://orcid.org/0000-0002-1280-4458>

Анотація. Мікобактеріози тварин, на відміну від таких у людей, внаслідок різних причин не перебувають в полі уваги ветеринарної науки і практики. Цей факт як і низка інших, зокрема зростання захворюваності людей на мікобактеріози, збудниками яких виступають нетуберкульозні мікобактерії, які причетні до сенсibiliзації організму продуктивних тварин, є об'єктивним, хоча й непрямим свідченням існуючої проблеми мікобактеріозів у

ветеринарній медицині. В процесі виконання роботи використано порівняльно-географічний та епізоотологічний методи аналізу. Результати, отримані з публікацій науковців різних країн світу, дали змогу узагальнити та об'єктивно оцінити напруженість епізоотичної ситуації щодо мікобактеріозів тварин у світі та в Україні, виявити низку особливостей інфекційного та епізоотичного процесів, а також встановити домінуючі етіопатогенетичні критерії цієї інфекції в популяціях тварин. Мікобактеріози тварин є предметом детальних наукових досліджень у більшості країн світу оскільки це захворювання має значне поширення серед продуктивних і непродуктивних (домашніх і диких) тварин, іхтіофауни морських і річкових водойм. Встановлено, що найчастіше як збудники мікобактеріозів виступають такі види нетуберкульозних мікобактерій як *Mycobacterium avium complex*, *M. abscessus complex*, *M. kansasii*, і *M. fortuitum* та низка інших. В Україні мікобактеріозам тварин не приділяється належна увага, не зважаючи на те, що параалергічні реакції на туберкулін у великої рогатої худоби призводять до ускладнення діагностики туберкульозної інфекції. Вони є причиною значних збитків в молочному скотарстві через вибраковку та забій високопродуктивних корів. Виявлення етіопатогенетичних закономірностей параалергічних реакцій на туберкулін у продуктивних тварин – запорука ефективного контролю та управління епізоотичним процесом за мікобактеріозу тварин та зменшення біоризиків інфікування людей нетуберкульозними мікобактеріями через продукти харчування тваринного походження

Ключові слова: туберкульоз; туберкулін; алерген атипових мікобактерій; епізоотичний процес; інфекційний процес; алергічні реакції; діагностика



2024. Volume 15, No. 2. P. 27-45

Received: 05.02.2024 Revised: 16.04.2024 Accepted: 29.05.2024

DOI: 10.31548/veterinary2.2024.27

UDC 636.09:615.2

Scientific substantiation of the use of iron-containing drugs in veterinary medicine

Iryna Derkach*

Doctor of Veterinary Sciences, Associate Professor
National University of Life and Environmental Sciences of Ukraine
03041, 15 Heroiv Oborony Str., Kyiv, Ukraine
<https://orcid.org/0000-0002-0149-7923>

Volodymyr Dukhnitsky

Doctor of Veterinary Sciences, Professor
National University of Life and Environmental Sciences of Ukraine
03041, 15 Heroiv Oborony Str., Kyiv, Ukraine
<https://orcid.org/0000-0002-9670-1244>

Igor Fritsky

Doctor of Chemistry Sciences, Professor
Taras Shevchenko National University of Kyiv
01033, 64 Volodymyrska Str., Kyiv, Ukraine
<https://orcid.org/0000-0002-1092-8035>

Maksym Plutenko

PhD in Chemistry Sciences, Researcher
Taras Shevchenko National University of Kyiv
01033, 64 Volodymyrska Str., Kyiv, Ukraine
<https://orcid.org/0000-0002-9369-0711>

Maksym Kohutych

Student
National University of Life and Environmental Sciences of Ukraine
03041, 15 Heroiv Oborony Str., Kyiv, Ukraine
<https://orcid.org/0009-0008-2659-4616>

Abstract. The relevance of this study is conditioned by the need to summarise current knowledge about the use of iron-containing drugs in veterinary medicine and their scientific substantiation. The purpose of this study was to analyse the results of the latest experimental data presented in

Suggested Citation:

Derkach, I., Dukhnitsky, V., Fritsky, I., Plutenko, M., & Kohutych, M. (2024). Scientific substantiation of the use of iron-containing drugs in veterinary medicine. *Ukrainian Journal of Veterinary Sciences*, 15(2), 27-45. doi: 10.31548/veterinary2.2024.27.

*Corresponding author



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the literature on the pharmacological and toxicological properties of iron-containing drugs and the specific features of their use in veterinary medicine. The study employed the method of literature analysis with the formulation of relevant substantiated conclusions and their generalisation or comparison. Iron-containing medicines are particularly relevant in modern veterinary medicine for the prevention of iron deficiency anaemia in piglets. The use of iron dextran preparations is a scientifically sound and necessary preventive measure in modern pig production. Furthermore, the study covered new approaches to the development of effective antianaemic drugs for animals of other species, including calves. The study also covered the controversial issues of the use of iron-containing drugs in pregnant females for the prevention of iron deficiency in animals born from them. The analysis of the literature data presented in this study shows a prominent degree of scientific interest in the study of pharmacological and toxicological properties of iron in high valences (specifically, ferrous iron in clathrochelate form) with a view to their use in medicine. The conducted literature review suggests the multidirectional use of iron-containing drugs in various dosage forms in veterinary medicine. The study identified the key trends in the pharmaceutical market of iron-containing drugs registered in Ukraine. In recent years, there has been a growing body of scientifically based evidence indicating the effectiveness of iron-dextran complex in combination with micro- and macronutrients and vitamins. Currently, the use of iron-containing drugs in the world's veterinary practice, considering their scientifically and experimentally proven pharmacological properties, is extremely promising. The materials of this study are of practical value for expanding the possibilities of using new forms of iron in veterinary medicine

Keywords: iron; iron deficiency; anaemia; piglets; calves; prevention

Introduction

Iron is one of the most abundant metals in nature. It is well known that as a chemical element, it can change its valence. At present, iron in low valence forms, i.e., the second and third, is well-researched. The properties of high-valent iron are being actively investigated in Ukraine and worldwide (Derkach, 2023; Plutenko, 2024).

In a living organism, the biological role of iron correlates with the physiological role of iron-containing compounds that ensure the body's vital functions. Thus, the trace element iron is a necessary structural part of haemoglobin, iron-containing and iron-dependent enzymes that affect physiological and biochemical processes in the body and cell functioning. It is natural that a lack or excess of this trace element provokes the development of pathological conditions, and with a prolonged

imbalance in its content, diseases occur that can have fatal consequences. M. Morales & X. Xue (2021) prove the correlation between iron levels in the body and cell proliferation in cancer.

According to recent publications, hepcidin plays a crucial role in iron metabolism in animals as a humoral factor, with an increase in the trace element's content stimulating hepcidin synthesis in the liver, which reduces iron absorption in the intestine and its transport into the bloodstream. Conversely, a decrease in iron absorption in the intestine leads to inhibition of hepcidin synthesis and, as a result, restoration of iron absorption. This hormone, which is a 25-amino acid peptide, affects the absorption and release of iron from macrophages during recycling from red blood cells. Hepcidin can also block iron transport in the placenta,

epithelium, and other tissues (Nemeth & Ganz, 2021). Under anaemic and hypoxic conditions, hepsidin gene expression decreases, while iron absorption from macrophages and the intestine increases (Vydyborets & Borysenko, 2019). According to J.H. Joachim & K.J. Mehta (2022), hepcidin, as a hormone that maintains systemic iron homeostasis, is involved in the development of hepatocellular carcinoma, one of the most common causes of death in cancer.

In veterinary medicine, the use of iron-containing products is particularly relevant in pig farming. This is primarily due to the risk of iron deficiency anaemia in piglets. Animals of this species have a biological predisposition to develop iron deficiency during the suckling period. First of all, this is conditioned by the fact that of all farm animals, they are born the most immature; their growth is comparatively quite intensive, which precedes the development of erythrocytopoiesis in their body. Inhibition of erythrocytopoiesis in the spleen and liver occurs against the background of activation of the erythropoietic capacity of the bone marrow. The consequence is that during this period, the piglet's body does not sufficiently ensure the production of red blood cells and the synthesis of haemoglobin (Derkach, 2023). According to M. Todorov *et al.* (2023), the complex use of iron-containing and tonic preparations in the case of rehabilitation and treatment measures in piglets is more effective than the conventional scheme used on farms for the treatment of weakened and anaemic piglets.

However, iron deficiency also develops in young animals of other species, and in adult animals it is a symptom of certain diseases or poisoning (Paslawski *et al.*, 2021). According to the latest data, haemotransfusion is effective in the treatment of anaemia in cats (Malyuk *et al.*, 2022). The development of new or improvement of current preventive measures against the occurrence and development of iron

deficiency in animals of different species stays relevant. Moreover, new properties of iron in high valence forms are being investigated with a view to their application in medicine.

The purpose of this study was to analyse the literature data on the findings of modern scientific research on the pharmacological and toxicological properties of iron-containing medicines and promising areas of their use in veterinary medicine, as well as their range in the pharmaceutical market of veterinary medicines in Ukraine. The study was conducted using widely recognised scientific methods, including search, observation, analysis, comparison, systematisation, and comparison with the findings of other studies, description, and a systematic approach. The search for and processing of scientific information was carried out through a literature review, which helped to identify key trends and gaps in existing knowledge. Observations of facts and phenomena were an essential component of obtaining concrete data and formulating assumptions. The analysis and comparison of the findings with the results of other studies helped to identify trends, confirmation or contradiction of the findings. Systematisation and structuring of information was important for the logical presentation of data. The description and systematic approach helped to put the research findings in context, contributing to the field and facilitating further research.

Historical background on the use of anti-anaemic drugs in veterinary medicine

As noted by H. Saito (2014), the trace element iron is important for the animal body, while its metabolism, according to recent data, is regulated primarily by the hormone hepsidin (Starzyński *et al.*, 2013; Machalová Šišková *et al.*, 2016; Sangkhae & Nemeth, 2017). We share the opinion of researchers who believe that in

veterinary medicine, iron deficiency poses the greatest risks to newborn piglets.

Iron deficiency anaemia is a separate nosological disease of suckling pigs. In animals of this species, it develops from 5-7 days of life, and a month after the birth of piglets, it manifests itself to the maximum. The main symptoms of this disease are a decrease in the number of red blood cells and/or a decrease in the content of haemoglobin per unit volume of blood; metabolic disorders, hypoxia, growth retardation, decreased resistance to other diseases, and a high mortality rate (Antonyak, 2002; Levchenko *et al.*, 2015).

According to primary sources, the first attempts to prevent anaemia in piglets were based on feeding newborn piglets red clay and watering them with solutions of iron and copper sulphates. With the development of pharmaceuticals in the 20th century, preventive measures were associated with the use (first enteral and later parenteral) of anti-anaemic drugs containing low-valent iron.

Thus, to prevent iron deficiency anaemia, it was recommended to use salts of iron, manganese, copper, and cobalt; licks with ferrous fumarate; chalk-based iron-containing briquettes; highly dispersed paste, granular feed with ferric glycerophosphate in piglet feeding (Ulyzko & Todorov, 2014). Among other options available were foal blood, protein extract of ruminant liver and spleen using the Filatov method. Generally, it was believed that the introduction of proteins, which can be sourced from vegetable or animal proteins (soy protein, albumin, gelatin, casein, etc.), has a positive effect. Liver products and immunostimulants were offered as anti-anaemic drugs. Subsequently, organic/inorganic and complex iron-containing compounds were widely used: ferrous sulphate (II) preparations for oral administration, ferrous dextran (III) preparations for enteral and parenteral administration. In general, from

the 1930s to the present day, anti-anaemic iron-containing drugs are generally divided into those used orally (monocompounds of iron salts, their combinations with cobalt and copper salts, vitamins (Svoboda & Pistkova, 2018), and those used parenterally (complex iron-containing compounds of iron with dextran, trace elements, vitamins).

As the range of iron-containing products has expanded, researchers have tried to develop classifications based on various criteria. Thus, according to one classification, this pharmacological group includes iron (II) medicinal products; iron (III) medicinal products; inorganic medicinal products; organic medicinal products. Therewith, inorganic compounds are represented by iron (II) sulphate and iron (III) chloride, while organic compounds are iron (II) gluconate, fumarate, xylitol, sorbitol, and iron (II) dextran, dextrin, and iron (III) ferroprotein (Troshin & Nechaeva, 2006).

According to the literature; researchers have investigated the use of iron-containing drugs in various dosage forms and with different routes of administration, including aerosolisation; oral administration in the form of powder (Grushanska, 2005), pastes (Ulyzko & Todorov, 2014), etc. Many journals indicate that they used to treat the sow's udder with a solution of ferrous sulphate (together with copper sulphate and cobalt chloride).

Use of iron-containing anti-anaemic drugs in suckling piglets

The conducted literature analysis suggests that currently the simplest and most effective way to prevent anaemia and treat animals with anaemia is to use iron-containing drugs in the neonatal period. However, the disadvantages of the injection route of administration are that this method involves non-physiological intake of iron in the animal's body, and high doses of the trace element are unsafe, as iron is a

heavy metal with the ability to accumulate and pro-oxidant properties (Boldt, 1999) and blocks phagocytic mononuclear cells in the form of haemosiderin (Levchenko *et al.*, 2015).

Notably, the method of oral administration of iron-containing medicines is natural. The use of iron in feed additives is considered a significant advantage for suckling piglets in terms of prevention of iron deficiency anaemia (Maes *et al.*, 2011; Starzyński *et al.*, 2013). R. Antileo *et al.* (2016) investigated the anti-anaemic efficacy of non-heme (NHI) and heme (HI) iron microparticles to develop a new oral iron supplement. It was found that iron deficiency anaemia in piglets can be prevented by administering an oral encapsulated NHI/HI supplement in three doses.

However, the authors of the present study share the opinion of scientists who believe that iron absorption is inhibited by inflammation and this method of treating anaemic animals is ineffective in case of metabolic disorders in their bodies. It is also well-known that inorganic iron-containing substances have poor absorption properties, which can result in dyspeptic conditions. T. Tuomainen *et al.* (1999) proved that, compared with the use of non-ionised iron-containing combination preparations, oral use of ferrous sulphate increases sensitivity to oxidation of plasma lipoproteins.

The study of the toxicological and pharmacological properties of new iron-containing antianaemic drugs stays relevant worldwide (Lipinski *et al.*, 2010; Geisser & Burckhardt, 2011; Cui *et al.*, 2018). Comparative studies of the efficacy of iron-dextran medicines in the prevention of anaemia and the treatment of patients with this pathology are relevant. Such medicinal products in a dose of 150-200 mg of iron per injection are recommended for intramuscular administration to piglets on the days 3-5 after birth (if necessary, repeated on the days 7-10). The advantage of using iron dextran compared

to iron salts is that it creates an iron depot in the body of newborn animals and minimises the risk of anaemia. The researchers found that the absolute weight gain in piglets treated with Bioferon during the first month of life was 880 g higher, and in piglets treated with Suifervit – 1,134 g compared to the control. At weaning, the average weight of piglets was 1.81 kg and 2.66 kg higher, respectively. Researchers attribute this to the fact that in the first case, Suifervit contains trace elements and vitamins (Prokopenko & Martynov, 2012).

Many researchers believe that iron-dextran preparations should be combined with trace elements (zinc, copper, cobalt) and vitamins (groups B, C), which will help stimulate haemocytopoiesis, as iron is absorbed by only 60-70% when administered parenterally. J.D. Cook & M.B. Reddy (2001) found that the use of ascorbic acid promotes haemocytopoiesis, as iron absorption increases by 2.9-3.5 times. Some scientists emphasised the pro-oxidant capacity of iron ions in high concentrations, which was considered a disadvantage of iron-dextran preparations. Therefore, combinations with cyanobalamin, tocopherol, and selenium were recommended. Therewith, developers of new iron-containing drugs have tried to reduce the iron content in complex anti-anaemic drugs (Vered, 2006; Danchuk *et al.*, 2017).

T.S. Tokarchuk & V.V. Danchuk (2017) found that intramuscular injection of a complex of zinc, iron, and germanium citrates twice together with vitamin E supplementation increased piglet body weight by 6.8%. The use of 2.5 mL of this combination of trace element citrates increased the haemoglobin concentration by 18.0% and 15.5%, respectively, and increased the number of red blood cells in piglets' blood on day 35 of life. V.B. Todoruk (2012) noted that the use of an iron-containing micronutrient composition (synergistic essential polynuclear complexes that exhibit a

biocoordination effect) in piglets of the experimental group led to an increase in haemoglobin concentration by 18% on day 8, by 20% on day 14, and by 17% on 32, while the number of red blood cells increased by 8% on days 8 and 14, and by 4% on day 32 compared to the control. Therewith, an increase in haematocrit by 6-8% was found throughout the experiment. Erythrocyte parameters were increased compared to the control: MCH by 11-15%, MCV/MCHC by 5-9%. The use of additional amounts of trace elements contributed to their accumulation in the piglets' body, specifically, on day 32 of life, the level of iron in the animals' blood increased by 53%.

V.S. Bityutskyi (2007) proposed the development of complex preparations containing iron in the form of cluster compounds stabilised by the carbohydrate component of micelles. The latter is an analogue of ferritin and a source of a non-toxic and easily accessible form of the trace element. Apart from the prosthetic groups of antioxidant enzymes, such medicines include chelated complexes of copper, zinc, and selenium. It was found that the administration of polymetal and polymetal-selenium to suckling piglets prevented iron deficiency anaemia in animals, as evidenced by positive changes in common diagnostic markers of iron deficiency in the experimental groups compared to the control. Thus, according to the findings of research by many authors, it was proved that a reliable anti-anaemic effect in suckling piglets was observed when iron was combined with other trace elements, vitamins, and antioxidants.

In previous studies by I. Derkach (2023), a comprehensive solution to this scientific and practical problem was presented, which makes provision for the investigation of the anti-anaemic activity of iron in valence IV. S. Tomyň *et al.* (2017) first reported clathrochelate iron compounds in valence IV. The physicochemical properties of these compounds were

actively studied: the high stability of iron (IV) clathrochelate was found to be primarily conditioned by the fact that metal ions in this compound are "packed" in an organic matrix, from which the metal is released gradually *in vivo* as the substance biodegrades. It was predicted that the use of medicinal products based on iron (IV) clathrochelate would minimise the undesirable effects associated with the toxicity of aquariums and aquacomplexes of low-valent iron. Preclinical studies have established that iron (IV) in clathrochelate form corresponds to Hazard Class III according to the classification of chemicals by hazard and to Toxicity Class IV as a low-toxic substance. The studied tetravalent iron exhibited weakly expressed cumulative properties, and no irritant or allergic effects were observed. Clinical studies of iron (IV) clathrochelate and its use in newborn piglets have shown an anti-anaemic effect, which is confirmed by high levels of haemoglobin, haematocrit, and red blood cell count (Derkach, 2023).

Many publications are devoted to the study of the effectiveness of nanocompounds in medicine. The use of iron nanocompounds is also promising in veterinary medicine. Such drugs in low doses have a pronounced pharmacological effect. Ukrainian researchers have conducted a range of studies of the combination of iron nanopreparation and brovapherone-100. It was found that the iron content in the blood plasma of suckling piglets increased during 20 days of life compared to piglets that were administered these drugs separately. It was also proved that piglets from the experimental groups were more active and competitive than those from the higher lactation groups. The use of iron nanochelates contributed to a dose-dependent decrease in cortisol concentration, an increase in T3 and insulin concentration in piglets' blood, which increased stress tolerance,

productivity, and resistance of animals. It was found that the combined administration of iron nanopreparation and iron dextran reduced the prooxidant effect, while increasing the activity of catalase and glutathione peroxidase (Danchuk *et al.*, 2017). Thus, despite the long history of research on piglet iron deficiency anaemia, the development of drugs for its prevention and treatment of sick animals, this issue is still relevant today.

Use of iron-containing anti-anaemic drugs in sows

The issue of using iron-containing preparations in pregnant sows to prevent anaemia in piglets born from them is currently under debate. Some scientists believed that the administration of iron-containing medicines to pregnant and lactating animals would ensure reliable iron reserves in the liver and blood of suckling piglets, while others believed that such preventive measures would not have a reliable preventive effect.

L.Ya. Bozhik (2009) noted that feeding sows with ferrous methionate in doses of 0.7 mg/kg body weight for 30 days before farrowing and 1.4 mg/kg before weaning would prevent the development of anaemia in piglets and have a positive effect on the physiological state of the sow's body and increase the count of red blood cells and haemoglobin content in their blood by 12.2% and 32.4%, respectively, compared to the control. Therewith, an increase in the count of T- and B-lymphocytes and immunoglobulin content was observed by 12.4%, 16.7%, and 19.9%, respectively; in sow colostrum, the level of total protein increased by 17.0% and immunoglobulins by 20.0%.

S. Bhattarai *et al.* (2019) investigated the relationship between haemoglobin levels in sows and piglets during farrowing, as well as the correlation between sow haemoglobin levels and the probability of foetal death. The re-

searchers found that the average haemoglobin content in the blood of sows and piglets was 106.9 ± 12.2 g/L and 124.4 ± 19.9 g/L, respectively. There is also a positive correlation between these indicators ($P=0.058$). On the other hand, the probability of dead piglets is negatively related to sow haemoglobin content ($P=0.021$). The findings obtained by the researchers suggest that the haemoglobin content in the blood of newborn piglets can be increased and still-birth rates reduced as a result of increasing the haemoglobin level in the blood of sows.

The results obtained by the researchers when using a solution of iron (IV) clathrocholate to farrowing sows 14 and 7 days before farrowing are of great significance. Studies have not observed any stillbirths or iron deficiency anaemia during the critical period of piglet rearing for the development of iron deficiency. The pharmacokinetic properties of iron (IV) clathrocholate administered to gestating sows were characterised by the ability of iron to penetrate the placenta and be excreted in colostrum. The above suggests the antianaemic activity of iron (IV) clathrocholate, and therefore it was proposed for the prevention of iron deficiency anaemia in piglets in production conditions: intramuscular injection of 10% solution of ferrous clathrocholate (IV) to pregnant sows at a dose of 10 mL 14 and 7 days before expected farrowing, which will ensure transplacental iron intake into the foetus; intramuscular injection of a 10% solution of iron (IV) clathrocholate at a dose of 10 mL and cyanocobalamin at a dose of 500 µg in the form of a solution 14 and 7 days before expected farrowing to stimulate erythropoietic function in pregnant sows and piglets born from them (Derkach, 2023). Thus, the opinion of researchers who consider the prevention of iron deficiency anaemia in piglets, which involves the use of iron-containing drugs in farrowing sows, to be effective, is relevant.

Use of iron-containing anti-anaemic drugs in calves

It is well-known that iron deficiency develops in young ruminants and other species, while in adult animals it is a symptom of various pathologies. Therefore, there are many reports in the literature on the effectiveness of various iron-containing drugs for the prevention of anaemia in other species of animals or the treatment of already anaemic animals.

According to V.M. Sokolyuk *et al.* (1995), it was proved that 30% of calves under 3 days of age suffer from anaemia, 35.4% – at 10 days of age, and 33.6% of these animals – at 20-40 days of age. The results of the clinical examination and laboratory analysis of blood serum for bilirubin and trace elements showed that at 1-3 days of age, calves had hypoplastic nutritional deficiency anaemia, which the researchers attributed to unsatisfactory feeding of dry cows. In older calves, myelotoxic anaemia was also observed, which was explained by the toxic effect on the bone marrow of products of impaired metabolism in diarrhoea syndrome. Calf anaemia was characterised by a marked decrease in haemoglobin content compared with changes in the number of red blood cells. Therefore, according to various classifications of anaemia, these animals showed hypochromic and microcytic anaemia. The hemoglobin content in the blood of sick 10-day-old calves was 77 ± 1.2 g/L (105 ± 1.2 g/L in healthy animals), the number of red blood cells, respectively, was 6.1 ± 0.1 T/L compared to 6.5 ± 0.15 T/L in healthy animals. The researchers pointed out that the largest number of calves with anaemia was observed in the last months of the year, in winter and early spring, while in summer the number of such cases decreased by 2-4 times. Thus, a critical decrease in the intensity of hemocytopoiesis occurred in the autumn-winter and spring periods. Therefore, it was in the autumn-winter and spring seasons that the use

of drugs to stimulate blood formation in calves was recommended.

Whole milk is known to contain low levels of iron, and therefore studies of iron deficiency anaemia in dairy calves fed whole milk are relevant. J. Allan *et al.* (2020) evaluated the effect of iron supplementation on calf growth rate and blood haemoglobin levels. It was found that iron caused an average increase in growth rate by 78 g/day (standard deviation 18 g/day) in calves of the experimental groups compared to the control. There was also a considerable increase in haemoglobin levels in the blood of calves aged 6 weeks, with haemoglobin levels in animals not receiving iron decreasing by an average of 12.1 g/L. In contrast, calves with higher growth rates in the first 6 weeks were also more likely to have low haemoglobin levels. There were differences in animal growth rates and haemoglobin levels on each farm. However, generally, on all farms, the level of iron in the body of calves affected their daily weight gain during the first 6 weeks. The researchers suggested that the magnitude of the effect depended on the farm, as there was a marked difference between the variations in animal growth rates across farms. The researchers proposed iron injections that led to a considerable increase in haemoglobin levels after 6 weeks, which prevented its decline during the first 6 weeks of life. The use of systemic iron supplements has been shown to have a positive effect. Therefore, considering the current industry recommendations to feed larger volumes of milk over a longer period of time, the researchers suggested systemic iron supplementation for calves fed whole milk. In the future, it was planned to establish a link between early iron deficiency and long-term growth rates of calves, as well as to investigate the relationship with their morbidity before weaning.

J. Joerling & K. Doll (2019) found no direct correlation between serum ferritin and serum

iron. Comparing calves' dairy diets; it was found that reduced levels of ferritin in the blood serum of calves were observed more often in those animals fed milk replacer compared to calves fed whole milk. The findings obtained by the researchers suggest that the determination of haemoglobin and serum iron content is limitedly suitable for the diagnosis of iron deficiency in calves. Serum ferritin was considered more informative, as serum iron levels are subject to physiological fluctuations, while iron deficiency can be caused by inflammation or neoplastic diseases.

V.I. Levchenko *et al.* (2015) investigated the effect of ferrolife on haemocytopoiesis in calves and piglets. They found that double administration of the drug in a dose of 4-5 mL to sick calves increased the haemoglobin content by 20.8%, iron concentration – by 23.5%, red blood cell count – by 31.4% and haematocrit value – by 8.6%; no negative effect on the albumin synthesising function of the liver was observed.

According to the results of studies by many researchers, it was proved that the iron contained in milk replacer meets only the basic needs of this trace element for erythrocytopoiesis. R. Kupczyński *et al.* (2017) established the effect of feeding a protein-iron complex on productivity and iron metabolism, haematological parameters, antioxidant and immune status within 35 days after calf birth. The preparation of the complex involved the enzymatic hydrolysis of milk casein and the addition of ferric chloride to the hydrolysate and lyophilisate. Therewith, experimental groups of calves were created that received low and high doses of iron as part of a protein-iron complex (10 g/day and 20 g/day, respectively) and a control group. Dietary supplements containing a lower concentration of iron had a significant positive effect on the metabolism of the trace element, while a higher dose of concentrate led to an increase in total iron binding capacity,

transferrin saturation and a decrease in unsaturated iron binding capacity, which indicated iron overload. Furthermore, treatment with lower doses of iron noticeably increased antioxidant parameters, mainly the overall antioxidant status of the body and the activity of blood glutathione peroxidase. At higher doses of iron in the protein-iron complex, a lower overall antioxidant status was observed. Concentrations of IgG, IgM, insulin, glucose, TNF α , and IGF-1 stayed unchanged in any of the groups after supplementation. The researchers stressed that in practice, the use of protein-iron complex concentrate requires accommodating for the iron content of milk substitutes and other feeds. Only a lower dose of the protein-iron complex had a positive effect on the immune and antioxidant status of the body. The authors plan to focus further research on the investigation of iron concentration in the liver, as well as the relationship between hepsidin-ferroportin and cytotoxicity after adding a protein-iron complex to calf diets.

A. Budny-Walczak *et al.* (2023) studied the effect of protein-iron complex on productivity and iron metabolism, haematological and biochemical parameters when fed whole milk until weaning. The Polish Holstein cattle were divided into a control group, where they were fed whole milk, and an experimental group, where calves received an iron supplement in milk at a dose of 16 g/day. To determine the production performance, calves were weighed at 7 and 42 days of life, and changes in such production parameters as average weight gain, feed conversion rate and growth rate were analysed. Blood was also taken from the jugular vein on days 7, 14, 28, and 42 of life. It was found that the average daily body weight gain, growth intensity and feed conversion rate were the highest in calves of the experimental group. The addition of a protein-iron complex had a substantial effect on iron metabolism in the experimental group of animals.

Doctors described the latest drugs containing the iron complex trimaltol (international non-proprietary name: ferrimaltol), which was developed, synthesised, and tested *in vitro* and *in vivo* in 1980-1981 after the discovery of a new class of alpha-keto-hydroxy-heteroaromatic iron chelators. According to the findings of relevant studies, it was established that natural and synthetic lipophilic chelators, including maltol, formed stable iron complexes and transferred it across cell membranes, as well as contributed to an increase in the intensity of iron absorption in animals (Kontoghiorghe *et al.*, 2021). Although the risks of developing iron deficiency anaemia in animals of other species at a young age are much lower compared to suckling pigs, their study is also relevant, and the scheme of preventive measures and treatment of sick animals needs to be improved considering modern world developments in medicine.

Iron-containing drugs in obstetric and gynaecological veterinary practice

Considering the significance of iron for all living things, apart from the use of iron-containing products for the prevention of anaemia, other areas of application of iron-containing products for various pathologies in animals, including obstetric and gynaecological veterinary practice, are being investigated.

V.Y. Stefanyk *et al.* (2017) proved that the effectiveness of intrauterine administration of suppositories containing iron nanoparticles after calving and aftermath of litter stimulated antioxidant functions in the body of cows, contributed to a decrease in the content of lipid peroxidation products. I.B. Kobylukh *et al.* (2017) found that after the application of suppositories with iron nanoparticles to cows (intrauterine after the afterbirth), the content of immunoglobulins of class A (3.9 times),

immunoglobulins of class M (2.8 times) in the blood serum significantly increased, while the content of immunoglobulins of class G decreased (4 times). The duration of the service period was reduced by 14 days ($P < 0.05$), and the insemination index decreased by 0.2 units compared to the control.

C.M. Oliveira *et al.* (2012) proposed new methods for studying the placental transition using iron and other nutrients necessary for the survival and maintenance of the embryonic foetus until birth. According to the classification of placental types among animals (cattle, small ruminants, dogs, and cats), iron transfer across the placenta can occur by transferrin-associated absorption through the outer surface of the trophoblast in direct contact with the mother's circulating blood; erythrocyte uptake by the chorionic epithelium in direct contact with a pool of blood extracted from haemotophages; absorption by the chorionic epithelium in direct contact with iron-rich secretions from the endometrial glands and absorption by blood extracts on the surface of the mother and foetus with subsequent phagocytosis of erythrocytes by trophoblast cells. The function of erythrophagocytosis observed after extravasation of blood into the maternal-foetal interface has not been determined in several species. The researchers suggested that iron is transferred to the foetus through trophoblastic erythrophagocytosis in the haemophagelial region of the placenta, as well as in the glands of the endometrium.

Thus, the literature presents the findings of studies confirming the effectiveness of iron-containing preparations for obstetric and gynaecological pathologies. However, this issue needs to be investigated more extensively to develop new drugs in relevant dosage forms, register them on the pharmaceutical market, and introduce them into veterinary practice.

Analysis of the pharmaceutical market of iron-containing drugs registered in Ukraine

Currently, colloidal solutions of ferric hydroxides in low-molecular-weight glucose polymers (dextrans) are particularly effective in iron deficiency. In modern veterinary practice, they are administered to newborn animals (mainly piglets and calves) in the first days of life to prevent iron deficiency anaemia and ensure the creation of iron depots in the liver of young animals, as well as to fur-bearing animals when they are fed fish, etc.

Many publications cover the research on the pharmaceutical market for iron-containing drugs. Thus, previous publications have analysed the Ukrainian pharmaceutical market of anti-anaemic drugs for pigs in recent years (Derkach, 2023). In Ukraine, in 2017, the market was represented by 13 medicinal products in the group QB03A Anti-anaemic agents. Ukrainian pharmaceutical manufacturers (O.L.KAR-AgroZooVet-Service, Farmaton,

Brovapharma, Research and Experimental Production of the Institute of Epizootology, Biopharm and Vetsintez) provided 38% of the iron preparations (ATC-vet classification). At the same time, 62% of foreign pharmaceutical products in this group are represented by pharmaceutical companies from Estonia, Denmark, South Korea, Poland, France, and the Czech Republic. The medicines contained a dextran complex of ferrous hydroxide, which in some products was combined with vitamins, macro- and microelements. Moreover, the Ukrainian pharmaceutical market offers iron-containing feed additives, ready-made feeds and premixes for animals and poultry of various species in various forms.

In 2017-2022, the modern pharmaceutical market of veterinary drugs in Ukraine was sufficiently supplied with anti-anaemic drugs for pigs, but mostly imported drugs (Fig. 1).

The share of Ukrainian drugs in this group was higher in 2020, but in 2022 the share of imported drugs prevailed again (Table 1).

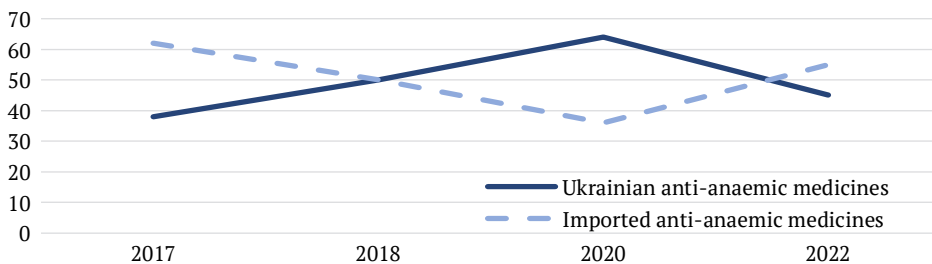


Figure 1. Trends in the domestic pharmaceutical market of anti-anaemic iron-containing drugs for pigs, 2017-2022

Source: I. Derkach (2023)

Table 1. Iron-containing drugs registered in Ukraine as of 01.01.2022

Drug name	Registration date	Registration valid until	Registration procedure	Manufacturer	Country of manufacture
Ferrum +	04.03.2021	03.03.2026	re-registration	Biotestlab	Ukraine
Brovaferan-100	24.12.2020	23.12.2025	re-registration	Brovapharma	
Ferrovvet +B12®	26.11.2019	25.11.2024	re-registration	Vet-synthesis	
Ferofort	12.04.2017	11.04.2022	registration	Fortis-pharma	
Bioferon Forte	26.11.2019	25.11.2024	registration	Biopharm	

Table 1. Continued

Drug name	Registration date	Registration valid until	Registration procedure	Manufacturer	Country of manufacture
Ferro 2000	07.07.2020	16.12.2023	changes	Dopharma France	France
Gafervit	07.07.2020	06.07.2025	re-registration	Bioveta, a.s	Czech Republic
Ferribion 10%	15.07.2019	14.07.2024	re-registration		
Intrafer-100 V12	07.07.2020	06.07.2025	re-registration	Interchem Verken "De Adelaar" Esti AS	Estonia
Intrafer-200 V12	04.03.2019	03.03.2024	re-registration		
Uniferon	28.02.2018	27.02.2023	re-registration	Pharmacosmos A/S	Denmark

Source: I. Derkach et al. (2023)

According to the analysis of the pharmaceutical market of iron-containing drugs, according to official data provided in the State Register of Veterinary Drugs, it was found that this group is currently represented by 11 anti-anaemic drugs out of 1,394 items registered in Ukraine as of 1 January 2024 (Official website of the State Service of Ukraine on Food Safety and Consumer Protection of People, n.d.). All medicinal products are solutions of trivalent iron in combination with dextran for injection. Of these, 5 drugs (45%) are of Ukrainian origin (manufacturers: Vetsintez LLC, JSC Biopharm LLC, Biotestlab LLC, German-Ukrainian Production Company Brovapharma LLC). Biopharm Pharmaceutical Company offers 2 iron-containing products: Bioferon and Bioferon Forte. The first preparation is a 10% solution of iron dextran, containing excipients phenol and purified water. 1 mL of Biopheron Forte contains 7.5% of the active ingredient ferrodextrin, as well as copper chloride, cobalt chloride, cyanocobalamin, phenol, and highly purified water. These medicinal products are intended for the treatment of animals with anaemia and for the prevention of iron deficiency anaemia in sows, piglets, calves, and lambs.

Ferrovit+B₁₂ (Ukraine) contains iron (III) hydroxide complex with low molecular weight dextran, which stimulates the hematopoietic system, increases haemoglobin levels, increases the number of red blood cells, replenishes iron deficiency, and increases animal productivity, and cyanocobalamin, which stimulates

hematopoiesis, activates creatine synthesis, lipid metabolism, methionine biosynthesis, and metabolic processes necessary for DNA synthesis. The half-life of iron from blood plasma is 5 hours. Therewith, small amounts of iron are excreted in the urine, while dextran is metabolised and excreted by the kidneys. The drug is intended for the prevention and treatment of iron deficiency anaemia in calves, foals, sows, lambs, puppies, and piglets. If necessary, the injection of the drug in an analogous dose is repeated in 10-12 days.

Brovapheran 100 (Ukraine) is a complex compound of ferric hydroxide and low-molecular-weight dextran. Its pharmacological effect is based on increasing the level of iron and haemoglobin, increasing the number of red blood cells in the body, stimulating the haematopoietic system and improving the safety of young livestock. The medicine is used for the treatment and prevention of iron deficiency anaemia in animals, especially piglets and calves. 1 mL of Ferrum+ (Ukraine) contains 100.0 mg of ferrous iron (in a complex with low molecular weight dextran), 5.0 µg of cyanocobalamin, 200.0 µg of folic acid; excipients: phenol and water for injection. The drug is recommended for the treatment of piglets, sows, calves, lambs, goats, dogs, fur-bearing animals with iron deficiency anaemia and for the prevention of the disease in case of blood loss and haemorrhagic diathesis.

According to the product leaflets, the contraindications were listed by Ukrainian manufacturers as follows: do not use in animals with

insufficient vitamin E and/or selenium in their bodies; animals with diarrhoea; in combination with tetracyclines; do not mix with other veterinary drugs, and do not use in animals with hypersensitivity to the drug's components. Among the warnings for use, it was noted that allergic reactions may occur, and in case of overdose, peripheral vascular collapse, which may result in depression, slowing/acceleration of the pulse, hypotension, cyanosis of the mucous membranes, ataxia, and coma. Cases of no symptoms of toxicity within 12-48 hours after administration of the drug were described, but later, pulmonary edema, vascular and motor collapse, cyanosis, acute liver failure, etc. suddenly developed. In case of an overdose, it is recommended to use the antidote Deferoxamine, which forms water-soluble chelated compounds with iron that are rapidly excreted by the kidneys. In rare cases, animals may die, specifically in breeds that are genetically sensitive to iron-containing drugs. There may be slight pigmentation of the tissues in the injection site, slight swelling that disappears in 2-3 days. Antacids slow down the absorption of iron. Some manufacturers indicated that after the first withdrawal from the vial, the drug should be used within 14 days (subject to aseptic withdrawal and storage in a dark place at 5-25°C), while others indicated that after opening the vial, the drug should be used within 24 hours and that it should not be frozen.

The medicines Intrafer-100 B₁₂, Intrafer-200 B₁₂ (Estonia), Feribion 10% (Czech Republic), Ferro 2000 (France), and Uniferon (Denmark) contain a complex compound of iron (III) hydroxide and cyanobalamin. These drugs are prescribed intramuscularly and subcutaneously for the prevention and treatment of calves and piglets (Ferro 2000 and Uniferon were recommended only for pigs, including piglets) with iron deficiency anaemia. Hafervit injectable (Czech Republic) contains ferrous

dextran, porcine serum immunoglobulin, thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, nicotinamide, calcium pantothenate, copper chloride, and cobalt chloride anhydrous. It is prescribed only for pigs.

According to the manufacturers' official instructions for use, iron in combination with low-molecular-weight dextran is a low-toxic anti-anaemic drug that reliably increases haemoglobin levels. After parenteral administration, iron accumulates in the tissues and is gradually released from the dextran complex, binding to proteins. Up to 60% of iron dextran is absorbed 3 days after administration, and up to 90% within 1-3 weeks.

Conclusions

Iron-containing medicines are particularly relevant in veterinary medicine for the prevention of iron deficiency anaemia in suckling piglets. To stimulate erythrocytopoiesis in the hematopoietic organs, animals of this species are administered iron-containing compounds on the days 3-5 after birth, the most commonly used of which are iron-dextran preparations worldwide. There are a range of advantages and disadvantages regarding the methods and forms of their application, but in the system of preventive measures for pig health, such prevention is generally accepted. The review analyses the various possibilities of using iron-containing drugs in veterinary medicine and describes new approaches to this with due regard for the development of modern medicine and pharmaceuticals. The study analysed the pharmaceutical market of iron-containing veterinary drugs registered in Ukraine in recent years. Notably, the share of relevant Ukrainian medicines is lower than that of imported pharmaceutical products, which should be a motivational task for Ukrainian producers. There is a general trend towards the combination of iron dextran with vitamins (especially

cyanobalamin), macro- and microelements in modern anti-anaemic iron-containing drugs. Ukrainian scientists have paid great attention to the study of the prooxidant effect of these drugs and the effect of selenium on lipid peroxidation and the antioxidant system in general. The literature provides data on the use of iron-containing compounds in pregnant females to provide microelements to piglets after farrowing. Some scientists proved that the formation of a reserve of the trace element in the liver/blood of newborn piglets and in colostrum/milk of sows can be achieved by using iron-containing preparations during pregnancy and lactation, although others have refuted this. For this reason, this prevention scheme has not been widely used in veterinary medicine. At the same time, the authors have already proved that in production conditions for the prevention of iron deficiency anaemia in piglets, intramuscular injection of ferrous clathrochelate (IV) solution to farrowing sows 14 and 7 days before the expected farrowing has a reliable preventive effect (provided transplacental intake of iron into the foetus)

and intramuscular injection of 10% solution of ferrous clathrochelate (IV) and cyanocobalamin 14 and 7 days before expected farrowing (stimulated erythrocytopoiesis in farrowing sows and piglets born from them).

The findings presented in this study are significant for clarifying the possibilities of using new iron-containing drugs in veterinary medicine. For instance, this is the case with iron-containing medicinal substances in which the trace element has high valence forms. Only their physicochemical properties are well-studied, and the issues of pharmacokinetics and pharmacodynamics require further in-depth research and development of a new scheme for the effective prevention of iron deficiency in animals, which we consider to be promising areas for further research in the context of the subject under study.

Acknowledgements

None.

Conflict of Interest

None.

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Наукове обґрунтування застосування залізовмісних препаратів у ветеринарній медицині

Ірина Деркач

Доктор ветеринарних наук, доцент
Національний університет біоресурсів і природокористування України
03041, вул. Героїв Оборони, 15, м. Київ, Україна
<https://orcid.org/0000-0002-0149-7923>

Володимир Духницький

Доктор ветеринарних наук, професор
Національний університет біоресурсів і природокористування України
03041, вул. Героїв Оборони, 15, м. Київ, Україна
<https://orcid.org/0000-0002-9670-1244>

Ігор Фрицький

Доктор хімічних наук, професор
Київський національний університет імені Тараса Шевченка
01033, вул. Володимирська, 60, м. Київ, Україна
<https://orcid.org/0000-0002-1092-8035>

Максим Плутенко

Кандидат хімічних наук, науковий співробітник
Київський національний університет імені Тараса Шевченка
01033, вул. Володимирська, 60, м. Київ, Україна
<https://orcid.org/0000-0002-9369-0711>

Максим Когутич

Студент
Національний університет біоресурсів і природокористування України
03041, вул. Героїв Оборони, 15, м. Київ, Україна
<https://orcid.org/0009-0008-2659-4616>

Анотація. Актуальність дослідження зумовлена необхідністю узагальнення сучасних уявлень про застосування залізовмісних лікарських засобів у ветеринарній медицині та їх наукового обґрунтування. Мета дослідження – провести аналіз результатів останніх експериментальних даних, представлених у літературі щодо фармако-токсикологічних властивостей залізовмісних препаратів та особливостей їх використання у ветеринарній медицині. Під час дослідження використано метод аналізу літературних джерел з формулюванням відповідних обґрунтованих висновків та їх узагальнення чи порівняння. Особливо актуальними залізовмісні лікарські засоби у сучасній ветеринарній медицині є за профілактики залізодефіцитної анемії у поросят. Застосування залізодекстанових препаратів є науково обґрунтованим і необхідним превентивним заходом у сучасному свинарстві. Крім того, у статті розкриваються нові підходи до розробки ефективних антианемічних препаратів тваринам інших видів, зокрема телятам. Розкрито дискусійні питання щодо застосування залізовмісних препаратів вагітним самкам з метою

профілактики залізодефіциту у народжених від них тварин. Аналіз літературних даних, представлений у статті, засвідчує високий ступінь наукового інтересу до вивчення фармако-токсикологічних властивостей заліза у високих валентностях (зокрема заліза чотирьохвалентного у клатрохелатній формі) з метою їх застосування у медицині. Наведений огляд літературних джерел засвідчує про багатовекторність застосовування у ветеринарній медицині залізовмісних лікарських речовин у різних лікарських формах. Встановлено основні тенденції на фармацевтичному ринку залізовмісних препаратів, зареєстрованих в Україні. Упродовж останніх років зростає кількість науково обґрунтованих доказів, які вказують на ефективність залізодекстранового комплексу в комбінації з мікро- та макроелементами і вітамінами. Нині у світовій ветеринарній практиці застосування залізовмісних препаратів із врахуванням їх науково обґрунтованих та експериментально доведених фармакологічних властивостей є надзвичайно перспективним. Матеріали статті становлять практичну цінність для розширення можливостей застосування нових форм заліза у ветеринарній медицині

Ключові слова: залізо; залізодефіцит; анемія; поросята; телята; профілактика



2024. Volume 15, No. 2. P. 46-65

Received: 13.02.2024 Revised: 23.04.2024 Accepted: 29.05.2024

DOI: 10.31548/veterinary2.2024.46

UDC 612.357.15; 612.357.32; 616.36-003.826

Bilirubin derivatives in bile, blood, and liver of rats upon correction of experimental fatty hepatosis

Josef Illek*

Doctor of Veterinary Sciences, Professor
University of Veterinary Sciences Brno
61242, 1946/1 Palackého Str., Brno, Czech Republic
<https://orcid.org/0000-0002-1374-7918>

Stanislav Veselskyi

Doctor of Biological Sciences, Senior Researcher
Taras Shevchenko National University of Kyiv
01601, 64/13 Volodymyrska Str., Kyiv, Ukraine
<https://orcid.org/0000-0001-9971-0333>

Andrii Pototskyi

Postgraduate Student
National University of Life and Environmental Sciences of Ukraine
03041, 15 Heroiv Oborony Str., Kyiv, Ukraine
<https://orcid.org/0000-0003-2500-6647>

Viktoriiia Gryshchenko

Doctor of Veterinary Sciences, Professor
National University of Life and Environmental Sciences of Ukraine
03041, 15 Heroiv Oborony Str., Kyiv, Ukraine
<https://orcid.org/0000-0003-0622-6345>

Yevdokiia Reshetnik

PhD in Biological Sciences
Educational and Scientific Center "Institute of Biology and Medicine"
Taras Shevchenko National University of Kyiv
01601, 64/13 Volodymyrska Str., Kyiv, Ukraine
<https://orcid.org/0000-0003-1084-842X>

Abstract. The relevance of this study lies in the lack of reliable information on the key aspects of the molecular mechanisms of fatty hepatosis, a common mammalian disease, and its dangerous

Suggested Citation:

Illek, J., Veselskyi, S., Pototskyi, A., Gryshchenko, V., & Reshetnik, Ye. (2024). Bilirubin derivatives in bile, blood, and liver of rats upon correction of experimental fatty hepatosis. *Ukrainian Journal of Veterinary Sciences*, 15(2), 46-65. doi: 10.31548/veterinary2.2024.46.

*Corresponding author



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complications, such as fibrosis, cirrhosis, and hepatocellular carcinoma. These circumstances substantially complicate the prompt diagnosis of this hepatopathology and reduce the effectiveness of treatment. The metabolism of the heme derivative bilirubin is unique and inherent in the liver, which undergoes transformation due to hepatocyte enzyme systems to form neutral compounds, such as glucurono-conjugates and sulphoconjugates, which undergoes changes and requires analysis in fatty hepatitis in animals. The aim of the study was to determine the characteristic changes in the content of unconjugated bilirubin and its sulphoconjugate, glucuronide, monoglucuronide, monoglucoside, and diglucuronide in the blood, bile, and liver of rats under the modelling of fatty hepatitis and the administration of rehabilitation therapy. For this, the thin-layer chromatography method was used. It was found that under tetracycline-induced fatty hepatitis in laboratory rats, the concentration of unconjugated bilirubin in the blood decreased by 39.3%, bilirubin glucuronide by 44.4%, and the total fraction of bilirubin monoglucuronide and monoglycoside by 78.9%. Oral administration of the milk phospholipid complex in the composition of the BAS "FLP-MD" to sick animals ensured the maintenance of these indicators at the control level. The content of bilirubin glucuronide and bilirubin diglucuronide decreased in the liver tissue of the treated rats. When these animals were administered a phospholipid-containing bioadditives, the level of unconjugated bilirubin in the liver tissue increased by 22.2%, but the content of its conjugated forms decreased. The content of all the studied bilirubin fractions decreased in the bile of sick rats during self-rehabilitation. The components of the BAS "FLP-MD" in fatty hepatitis in rats did not affect the reduction of unconjugated bilirubin in bile but eliminated the inhibitory effect of the modelled pathology on the content of all conjugated forms of bilirubin. The findings can be used as biochemical markers to monitor the pigment metabolism in the development of fatty hepatitis in mammals

Keywords: bile pigments; milk phospholipids; tetracycline hydrochloride; thin-layer chromatography; corrective therapy; fatty degeneration

Introduction

By the definition given in H. Tilg & M. Effenberger (2020) and Y. Liu *et al.* (2024), metabolic dysfunction-associated fatty liver disease (MAFLD) is a chronic progressive disease with a variable spectrum of clinical symptoms and varying severity of course: from steatosis to end-stage liver disease. Recent evidence suggests that total serum bilirubin levels are inversely correlated with the prevalence of metabolic syndrome, including metabolically associated fatty liver disease.

K. Moss *et al.* (2023) investigated the effect of bilirubin levels in patients on the complexity of the course and mortality stratified by the

severity of fatty liver disease. As a result, the researchers noted that higher levels of bilirubin in the blood of patients with fatty liver disease, which was diagnosed in patients in combination with other pathologies, were associated with a lower risk of mortality from cardiovascular disease and malignant tumours.

The data presented by H. Han *et al.* (2024) confirm that a moderate increase in total bilirubin has antioxidant and anti-inflammatory properties in many metabolic diseases, including fatty liver disease, and reduces the risk of all-cause mortality. It turns out that a high concentration of total bilirubin in the blood within the physiological range is associated

with a reduced risk of severe fatty liver disease, a reduced probability of its transformation into more severe forms of liver damage and the resulting mortality. These observations prompt the investigation of possible mechanisms of changes in pigment metabolism in metabolically associated fatty liver disease. Attention is also drawn to the question of whether metabolically related fatty liver disease is always accompanied by some increase in the level of total, unconjugated and conjugated bilirubin in the blood and other biological fluids and tissues. The answers to these questions can only be obtained by conducting comprehensive studies with analysis of clinical statistics and experimental modelling of fatty liver.

T. Yamamoto *et al.* (2021) suggest that a risk factor for the development of fatty liver disease is the overuse of antibiotics. The active use of antibiotics in animals has led to the detection of their residues in food derivatives such as milk and dairy products. G. Tarantino & V. Citro (2024) argue that antibiotic residues in the environment affect the intestinal flora of animals and humans, leading to a variety of metabolic changes that may ultimately contribute to the onset and progression of fatty liver. Therefore, the use of models of artificial reproduction of fatty liver in laboratory animals by using high-dose antibiotics in experimental veterinary medicine is of great practical significance. There are currently no proven and highly effective pharmacological treatments for fatty liver disease. The lack of properly validated pre-clinical studies using successful and relevant experimental disease models is a major obstacle to the development of clinical treatments. D.G. Buyco *et al.* (2021) and H. Wang *et al.* (2023) suggest that to find therapeutic approaches to fatty liver, the full range of metabolic disorders should be identified in model experiments and, based on the findings obtained, promising drugs of various origins should be tested.

A.J. McGlinchey *et al.* (2022) emphasise that the danger of fatty liver disease is that it is not amenable to drug therapy, and in many cases, it progresses to steatohepatitis with further progression to cirrhosis. M. Yin *et al.* (2021) prove the effectiveness and feasibility of using drugs of natural origin with minimal side effects on the body in fatty liver of various aetiologies. This is how phospholipid-containing medicines and phospholipid-containing food bioadditives have proven themselves. D. Wupperfeld *et al.* (2022) explain the effectiveness of phospholipid-containing agents by their hepatoprotective effect. M. Mitrovic *et al.* (2022) note that different sources of phospholipids (oilseeds, seafood, egg yolk, etc.) are used to produce phospholipid-containing agents with hepatoprotective properties, which leads to different properties and the corresponding effectiveness of the use of drugs derived from them.

V.A. Gryshchenko *et al.* (2023) point out that milk is a valuable source of phospholipid complexes, the unique phospholipid spectrum of which is inherent in mammals, and therefore is expected to be the most effective for the designing of preparations for the medication of animals. Researchers have confirmed a substantial positive effect of the biologically active supplement (BAS) FLP-MD, based on a complex of milk phospholipids, on lipid metabolism in animals with calf dyspepsia and toxic hepatitis.

Therefore, the purpose of this study was to determine the content of unconjugated bilirubin and its conjugated forms in blood, bile, and liver tissue in laboratory rats under artificial reproduction of fatty hepatosis, and in the case of corrective therapy based on a complex of milk phospholipids in the composition of the BAS “FLP-MD”.

Literature Review

According to A. De & A. Duseja (2022), fatty liver is characterised by the accumulation of lipid

vesicles in hepatocytes without pronounced changes resulting from inflammatory processes in the liver tissue – steatosis (fatty liver) or with lobular inflammation – steatohepatitis. Generally, fatty liver constitutes a wide range of structural and functional disorders, the pathogenesis of which does not involve hepatotropic viral infections, autoimmune processes, or clearly defined genetic risk factors.

According to the data presented in X. Shu *et al.* (2022), fatty degeneration of liver tissue is a fairly common hepatopathology in the world. It is believed that up to 25% of the world's population suffers from it, which is accompanied by hyperlipidemia, obesity, diabetes mellitus, and hypertension. At the same time, according to G. Tarantino & V. Citro (2024), all these disorders (obesity, insulin resistance, metabolic syndrome) are among the factors that provoke the development of fatty liver. N. Chalasani *et al.* (2018) associate the occurrence of this disease primarily with the development of metabolic syndrome and disorders of not only lipid metabolism but also closely related carbohydrate metabolism. H. Tilg & M. Effenberger (2020) and Y. Liu *et al.* (2024) point out the heterogeneous pathogenesis of fatty liver disease, in which metabolic disorder is the leading factor. Since fatty liver is associated with metabolic dysfunction, researchers have proposed to focus on identifying changes in various parts of metabolism in this pathology and to accelerate the search for new effective drugs and treatments.

Metabolic steatohepatitis is one of the hepatocellular diseases that can cause pigment metabolism disorders. T. Yamamoto *et al.* (2021) reported that in the case of severe hepatocellular necrosis, an increase in transaminase activity was observed, and hyperbilirubinaemia developed. In fatty liver disease, no jaundice was observed in the absence of inflammatory processes and significant disorders of bilirubin transport and biotransformation. As

summarised by K. Moss *et al.* (2023), an increase in blood bilirubin levels and a decrease in its content in bodily fluids may result from changes in various stages of its metabolism. Specifically, due to excessive production of bilirubin (pathological haemolysis), there is a disturbance in the liver's absorption of bilirubin from the blood and an increase in the level of unconjugated bilirubin, followed by a disturbance in its conjugation caused by a defect in UDP-glucuronosyltransferase, and bile clearance disorders with increased levels of direct bilirubin due to defects in the transport proteins of the channel (apical) domain of the hepatocyte plasma membrane or the inability of bile to reach the small intestine in case of bile duct obstruction. Liver damage caused by any cause and with varying degrees of severity reduces the number of functionally fit hepatocytes, which can impair the absorption of unconjugated bilirubin from the blood plasma and reduce bilirubin clearance through the bile ducts. Therefore, deviations from the normal content of bilirubin and its derivatives can be detected in blood and bile tests even in the absence of jaundice accompanying metabolically determined liver diseases. Over three decades ago, H.S. Kaufman *et al.* (1991) also reported that elevated bilirubin levels in bile may be another pathogenic factor in the formation of gallstones, even in the absence of cholesterol supersaturation of the hepatic secretion. In some models of experimental fatty liver, an increase in blood bilirubin levels was also detected.

M.P. Boychak *et al.* (2022) concluded that an increase in the concentration of conjugated bilirubin in the blood is associated with hepatocellular lesions, not just cholestasis. Whereas changes in unconjugated bilirubin are more indicative of the ability to synthesise less toxic forms of bile pigment metabolites even in critical conditions. Lipid peroxidation and oxidative stress are considered the leading factors in the

development of fatty hepatosis of various aetiologies. N. Sadasivam *et al.* (2022) claim that cytochrome P450 2E1 (CYP2E1), which also produces reactive oxygen species (H_2O_2 and O_2^-), is a provoking factor in the development of fatty liver disease, as inhibition of CYP2E1 has been proven to protect liver cells in disorders regulation of lipid metabolism and oxidative stress. According to the researchers, serum bilirubin can also affect the manifestation of cytoprotective and antioxidant phenomena in the absence of hepatopathology. Serum bilirubin levels are inversely correlated with insulin resistance and the development of diabetes mellitus and cardiovascular disease in patients with fatty hepatitis. At the same time, the correlation between serum bilirubin levels and fatty liver is still unclear. It was suggested that elevated levels of bilirubin and its metabolites in the blood serum can reduce oxidative stress, inflammation, and inhibit the development of fatty liver. However, as noted by X. Shu *et al.* (2022), there was no independent association between serum bilirubin and fatty liver infiltration in non-obese patients. Thus, there is an urgent need to determine the causal relationship between bilirubin metabolism and excretion and fatty hepatosis in randomised controlled trials.

According to D.O. Melnychuk *et al.* (2014), the effectiveness of the use of milk phospholipids in the composition of the BAS “FLP-MD” for the correction of lipid metabolism disorders in toxic hepatitis by eliminating the structural and functional destabilisation of hepatocytes, especially their membrane systems, was established. The established effectiveness of a bioadditive containing milk phospholipids (FLP-MD) is promising for veterinary medicine, including in the development of drug-induced hepatosis. As discussions continue on the involvement of pigment metabolism and fluctuations in the content of bilirubin and its derivatives in the mammalian body in the pathogenesis of fatty

liver disease, the question arises as to the specific features of changes in this metabolism when using milk phospholipids in the composition of the BAS “FLP-MD” in conditions of modelled fatty hepatosis.

Materials and Methods

A series of experimental studies was conducted at the scientific laboratories of the Faculty of Veterinary Medicine of the National University of Life and Environmental Sciences of Ukraine and the Educational and Research Centre “Institute of High Technologies” of Taras Shevchenko Kyiv National University in 2022-2023. To determine the effects of milk phospholipids in the BAS “FLP-MD” under conditions of simulated fatty hepatosis on the content of various forms of bilirubin: unconjugated bilirubin, bilirubin sulphate, bilirubin monoglucuronide, total bilirubin monoglucuronide and monoglucoside fractions, bilirubin diglucuronide in liver tissue, blood, and bile, an experimental study was conducted using male *Wistar* rats (32 animals) weighing 200 ± 50 g. Only males were used for the experimental studies because of the known sexual dimorphism of fatty liver disease in mammals (Martin-Grau & Monleon, 2023) and differences in clinical manifestation and prognosis in animals of different sexes. To weigh rats during the experiment, electronic scales ORION OS-0K22 (ORION ELECTRONICS LTD, Europe) were used.

The laboratory animals were maintained in a vivarium under standard state, with an indoor temperature of 22-24°C, 14-hour daylight hours, a standardised nutritious diet and free access to water. The experimental study was conducted according to the European convention for the protection of vertebrate animals used for research and other scientific purposes (1986) and the Law of Ukraine No. 3447-IV (2006). All surgical interventions required by the study plan in acute experiments were

performed according to the ARRIVE guidelines, following the guidelines of Council Directive 2010/63/EU (2010) on the protection of animals used in experiments.

The author's methodology (Gryshchenko *et al.*, 2019) was used to model fatty hepatosis in laboratory rats. For this, applying a soft silicone tubes, the animals were administered a 4% solution of tetracycline hydrochloride in the high dose: 250 mg/kg body weight daily intragastrically for 7 days. As a result, rats developed fatty hepatosis induced by high-dose tetracycline. Rats that were treated with intragastric administration of tetracycline hydrochloride were included in the "Self-rehabilitation" group (n=8). Animals of the "Correction" group (n=8) received an intragastric 1% solution of the BAS "FLP-MD", the main active ingredient of which is milk phospholipids, for 7 days before oral administration of the antibiotic and additionally for the next two days after its completion. The daily single dose of the BAS "FLP-MD" was 13.5 mg/kg body weight (Pat. 86516 UA, 2009). Animals that were administered intragastrically with the same volume of distilled water to the volume of antibiotic and bioadditive were in the "Control" group (n=8). A separate group of animals stayed "intact" in terms of antibiotic use but received milk phospholipids as part of the BAS "FLP-MD" (n=8). An essential component of the study was regular determination of the animals' body weight and recalculation of the dose of preparations according to its change during the experiment. In acute experiments on rats, bile samples were collected with a cannulated bile duct. The duration of the acute experiment was two hours. The day before the surgery experiment (acute experiment), we weighed the rats and deprived them of access to food. During the day before the acute experiment, animals of all experimental groups had free access to drinking water. The absence of feeding during the day before the acute experiment

was conditioned by the need to avoid possible reflex regulatory effects of feed intake on biliary function and blood chemistry.

To perform the surgical intervention in the acute experiment, rats were intraperitoneally injected with sodium thiopental at 7 µg/100 g of body weight, which ensured immobilisation and anaesthesia of the animals. The anaesthetic effect occurred 3-5 minutes after the drug injection. Subsequently, the abdominal wall of the experimental animal was cut along the white line of the abdomen. After that, we cannulated the animal's bile ducts using a special plastic tubes (cannulas) to which a micropipettes were attached. The cannula was fixed in the bile duct using ligatures. Bile samples were distributed into separate plastic epidurals every 30 minutes during the acute experiment. As a result of the one-and-a-half-hour acute experiment, three individual half-hour bile samples were taken from each rat at the end of the acute experiment, blood and liver tissue samples were taken for further determination of the content of bilirubin and its derivatives. In samples of liver tissue, bile and whole heparin stabilising blood, after extraction according to the method Pat. 99031324 UA, MPK A61B5/14, the concentration of free and conjugated forms of bilirubin was identified and determined according to the method under Utility model patent 41602 Ukraine, IPC G01N 33/52, G01N 33/72 (Garnyk, 2009). For this, 50 µL of stabilising aqueous solution was added to the liver, bile, or blood samples obtained in the acute experiment (50 µL each). The stabilising solution contained 5.0% urea and 0.5% ascorbic acid. Subsequently, butanol and acetone were added to the mixture of the biological sample and stabilising solution in the following volume ratio: 2:2:7. The mixture was intensively mixed and centrifuged for 10 minutes at 3,000 rpm. A laboratory centrifuge OPN-8 (Ukraine) was used to centrifuge the resulting mixture. Acetone and butanol

were passively evaporated from the supernatant. Subsequently, 5 µL of the aqueous component from the biological sample extract was applied 2-4 times to pre-marked Silufol chromatographic plates (Czech Republic). The chromatographic separation of pigments extracted according to the method described above is also possible on FN-16 chromatographic paper from Filtrak (Czech Republic). The chromatographic separation of pigments from the processed liver, bile, or blood samples was performed in the chromatography chamber. For this, the side walls of the chromatography chamber were preliminarily lined with filter paper and a mixture of solvents was poured into the chamber: amyl acetic acid ester, concentrated acetic acid, propanol, water, ethylene glycol in a volume ratio of 21:10:5:5:3. The chromatography plates or chromatography paper with the applied samples were placed in the chromatography chamber. After the separation into fractions was completed, solvent was extracted from the chromatograms in a fume hood. The finished chromatograms were previewed in the visible and ultraviolet regions of the spectrum using special light sources (chromatoscope and lamp type A-FC-301) to activate the fluorescence of pyrrole groups in bilirubin and its derivatives. For quantification, the chromatograms were stained using a glass laboratory sprayer to apply the modified diazo reagent: 10 mL of diazo solution No. 1 and 0.25 mL of diazo solution No. 2 with 1.0 mL of formic aldehyde added to the mixture. Densitometry of 5 separate fractions of different forms of bilirubin metabolites was performed in the ultraviolet and visible spectral range using a densitometer DO-1M (Ukraine). The methodology used for the identification and distribution of pigments in bile and blood helped to determine unconjugated bilirubin, bilirubin sulphate, bilirubin monoglucuronide, the total fraction of bilirubin monoglucuronide and monoglucoside, and bilirubin diglucuronide in both of these biological fluids.

The results of the study were statistically processed using Statistica 5.0 software (StatSoft Inc., USA). According to the recommendations of N.B. Filimonova *et al.* (2005) used the t-Student's test to determine the statistical reliability of differences between the values of indicators of pigment metabolism in the studied groups. To test the nature of the distribution for normality, the study employed the Shapiro-Wilk test offered by Statistica 5.0 software. It was found that the concentrations of all five fractions of bilirubin derivatives obtained in the experiment were normally distributed. Differences between two comparable indicators from two different samples were defined as statistically reliable at $P < 0.05$, $P < 0.01$, or $P < 0.001$.

Results and Discussion

To investigate the mechanisms of influence of the components of the BAS "FLP-MD" on the metabolism of bile pigments in the body of laboratory rats with artificial reproduction of fatty hepatitis, the content of bilirubin metabolites was determined in samples of blood, bile, and liver tissue. A study of the levels of various bilirubin metabolites in liver tissue, bile, and blood obtained from rats with artificially reproduced fatty hepatitis showed that both in the modelling of this hepatopathy and in case of using milk phospholipids in the composition of the "FLP-MD" bioadditive, the fractions of free and conjugated bilirubin underwent substantial quantitative changes.

The concentration of unconjugated bilirubin in the blood of animals with tetracycline-induced liver damage was 39.3% lower than the control value (Table 1). Oral administration of the milk phospholipid complex in the BAS "FLP-MD" to rats both under conditions of modelled pathology and in intact animals did not affect the concentration of unconjugated bilirubin in the blood, and therefore it did not differ from the control values.

Table 1. The content (mg%) of bilirubin derivatives in the blood of experimental rats under experimental conditions ($M \pm m$, $n = 8$)

Bile pigment fraction of blood	Animal group			
	“Control”	“Self-rehabilitation”	“Correction”	“Healthy + BAS “FLP-MD””
Bilirubin unconjugated	0.56 ± 0.16	$0.34 \pm 0.10^{\#}$	0.55 ± 0.06	0.64 ± 0.09
Bilirubin sulphate	0.0087 ± 0.0045	0.0042 ± 0.0027	0.0085 ± 0.0021	0.0105 ± 0.0024
Bilirubin glucuronide	0.054 ± 0.016	$0.030 \pm 0.010^{###}$	0.066 ± 0.007	0.057 ± 0.020
Bilirubin monoglucuronide and monoglucoside	0.019 ± 0.009	$0.004 \pm 0.002^{####}$	0.020 ± 0.006	0.019 ± 0.006
Bilirubin diglucuronide	0.067 ± 0.02	0.047 ± 0.02	0.060 ± 0.01	0.063 ± 0.01

Notes: * $P < 0.05$, ** $P < 0.01$, statistically significant differences between the indicators of the experimental groups (“Self-rehabilitation”, “Correction”, “Healthy + BAS “FLP-MD”” groups) and the control indicators (“Control” group); # $P < 0.05$, ### $P < 0.001$, the difference is significant between the values of indicators in the “Correction” and “Self-rehabilitation” groups

Source: developed by the author of this study

Thus, the administration of the phospholipid complex in the form of the BAS “FLP-MD” to rats did not cause significant changes in the concentration of unconjugated bilirubin in the blood and slightly reduced it in the bile in intact animals. In rats with artificially reproduced fatty hepatosis, the administration of the BAS “FLP-MD” had an effective effect in terms of complete recovery to the control values of unconjugated bilirubin concentration in the blood and partial recovery in bile. Therewith, the concentration of unconjugated bilirubin in the blood of animals with modelled fatty hepatosis was substantially lower (by 39.3%) than the control value.

In most cases of liver pathology, researchers observed hyperbilirubinaemia due to an increase in the level of its unconjugated form in the blood. As pointed out by S. Gazzin *et al.* (2017), unconjugated hyperbilirubinaemia is associated with increased bilirubin production, impaired delivery from the blood to hepatocytes, and improper functioning of enzyme systems that ensure pigment conjugation in hepatocytes. Generally, unconjugated bilirubin can bind to biomembranes, which changes their fluidity, affects mitochondrial respiration, and the production of reactive

oxygen species. Thus, this form of pigment acts on its own transport and enzyme systems, changing metabolism. The researchers emphasised that unconjugated hyperbilirubinaemia is not typical for fatty hepatosis, and its manifestations are recorded in the transition of steatosis to steatohepatitis with further progression of liver fibrosis. The experiment revealed that the level of unconjugated bilirubin in the blood of rats of the “Self-rehabilitation” group was characterised by a decrease compared to the control and the value of this indicator in the “Correction” group. Situations in which blood bilirubin concentrations are substantially reduced, as reported by Y. Aoki *et al.* (2022), also correlate with impaired renal function and pancreatic damage. In this regard, the authors of the publication emphasise the need for constant monitoring of the kidneys in case of low bilirubin levels. According to Y. Aoki *et al.* (2022), the search for therapeutic measures to normalise pathologically reduced levels of unconjugated bilirubin is a vital area of research to prevent hypobilirubinaemia-related disorders of body functions.

C.-C. Zhao *et al.* (2023) reported normal and slightly elevated levels of both unconjugated and conjugated bilirubin fractions in blood

plasma, which may suggest the anti-inflammatory and protective effects of unconjugated bilirubin and is an angioprotective factor. L. Vitek & C. Tiribelli (2021) noted that unconjugated bilirubin can be considered as a biologically active factor with antioxidant properties that reduces the risks of cardiovascular disease and atherosclerosis, metabolic syndrome and diabetes mellitus, autoimmune and neurodegenerative diseases. Bilirubin should be considered not only as a potentially dangerous end product of heme metabolism, but also as a kind of “yellow hormone” that affects metabolism, which requires thorough investigation. Conditions in which the level of bilirubin in the blood decreases can be dangerous for the course of many common diseases and increase the risk of complications. D.O. Melnychuk *et al.* (2014) observed a decrease in the content of unconjugated bilirubin (by 20%) in the whole blood of rats with simulated cadmium chloride injury at a dose corresponding to 1/50 LD₅₀.

We assume that the 39.3% decrease in the concentration of unconjugated bilirubin in the blood of diseased rats may be explained by the inhibition of albumin biosynthesis in hepatocyte damage and reduced haemoglobin production due to inhibition of the hematopoietic function of the red bone marrow under conditions of artificial reproduction of fatty hepatosis, as demonstrated in a previous study (Gryshchenko *et al.*, 2019).

The unconjugated bilirubin content stayed unchanged in the liver tissue of the “Self-rehabilitation” group rats (Table 2). On the contrary, the administration of the milk phospholipid complex in the BAS “FLP-MD” in intact animals caused a 27.6% decrease in the content of unconjugated bilirubin in liver tissue compared to the control. However, when using the BAS “FLP-MD” in animals with experimental fatty hepatosis, the concentration of unconjugated bilirubin in liver tissue was 22.2% higher than in the control.

Table 2. Content (mg%) of bile pigments in the liver tissue of experimental rats under experimental conditions (M ± m, n = 8)

Bile pigment fraction	Animal group			
	“Control”	“Self-rehabilitation”	“Correction”	“Healthy + BAS “FLP-MD””
Bilirubin unconjugated	3.69 ± 0.82	3.11 ± 0.51 ^{***}	4.51 ± 0.65 [*]	2.67 ± 0.56 ^{###}
Bilirubin sulphate	1.56 ± 0.44	1.78 ± 0.27 ^{***}	0.95 ± 0.16 ^{**}	1.77 ± 0.34 ^{###}
Bilirubin glucuronide	13.24 ± 1.93	10.50 ± 1.77 [#]	9.16 ± 0.74 ^{***}	13.16 ± 2.11 ^{###}
Bilirubin monoglucuronide and monoglucoside	1.79 ± 0.33	1.51 ± 0.21 ^{###}	0.99 ± 0.16 ^{***}	1.84 ± 0.29 ^{###}
Bilirubin diglucuronide	31.31 ± 4.76	25.09 ± 2.22 ^{***}	19.74 ± 2.06 ^{***}	27.81 ± 3.06

Notes: *P<0.05, **P<0.01, ***P<0.001, statistically significant differences between the indicators of the experimental groups (“Self-rehabilitation”, “Correction”, “Healthy + BAS “FLP-MD”” groups) and the control indicators (“Control” group); # P<0.05, ### P<0.001, the difference is significant between the values of indicators in the rats of the “Correction” and “Self-rehabilitation” groups or the “Healthy + BAS “FLP-MD”” group

Source: developed by the author of this study

The detected accumulation of unconjugated bilirubin in the liver tissue of the diseased animals of the “Correction” group may be associated with the intensification of pigment

transport from the blood to hepatocytes under the influence of milk phospholipids in the BAS “FLP-MD” due to the stimulation of albumin synthesis. We also assume that the components

of the BAS “FLP-MD” can stimulate the uptake of unconjugated bilirubin by hepatocytes in conditions of simulated fatty hepatosis, which is a significant therapeutic property of the phospholipid bioadditive under study and its ability to accelerate repair processes in cell membranes.

Unconjugated bilirubin was found in all three bile samples obtained from rats with modelled pathology at a concentration lower than in the first, second, and third control samples by 29.5%, 26.0% and 29.9%, respectively (Table 3).

Table 3. The content (mg%) of unconjugated bilirubin in the bile of experimental rats under experimental conditions ($M \pm m$, $n = 8$)

Experiment time, min	Animal group		
	“Control”	“Self-rehabilitation”	“Correction”
30	2.37 ± 0.33	1.67 ± 0.23***	1.90 ± 0.25**
60	2.30 ± 0.26	1.71 ± 0.27***	1.90 ± 0.26**
90	2.21 ± .27	1.55 ± 0.26***	1.81 ± 0.23*#

Notes: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, statistically significant differences between the indicators of the experimental groups (“Self-rehabilitation”, “Correction”, “Healthy + BAS “FLP-MD”“ groups) and the control indicators (“Control” group); # $P < 0.05$, the difference is significant between the values of the indicator in the “Correction” and “Self-rehabilitation” groups

Source: developed by the author of this study

When using the BAS “FLP-MD” in animals with modelled fatty hepatosis, the concentration of unconjugated bilirubin in all three bile samples remains lower than in control samples by 19.8%, 17.4%, 18.1%, respectively. However, when comparing the results in the “Self-rehabilitation” and “Correction” groups, it was found that the content of unconjugated bilirubin in the third bile sample of animals treated with a biologically active phospholipid

supplement was 17% higher than the content of unconjugated bilirubin in the third bile sample of rats with modelled fatty hepatosis (Table 3). Therewith, the concentration of bilirubin sulphate in all three bile samples obtained from rats with experimental hepatopathy was substantially lower compared to the first, second, and third samples in the control, by 20.8%, 22.6% and 36.0%, respectively (Table 4).

Table 4. The content (mg%) of bilirubin sulphate in the bile of experimental rats under experimental conditions ($M \pm m$, $n = 8$)

Experiment time, min	Group of rats		
	“Control”	“Self-rehabilitation”	“Correction”
30	1.06 ± 0.21	0.84 ± 0.13*	1.17 ± 0.18###
60	1.06 ± 0.18	0.82 ± 0.10**	1.00 ± 0.26###
90	1.00 ± 0.17	0.74 ± 0.11***	1.01 ± 0.23###

Notes: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.01$, statistically significant differences between the indicators of the experimental groups (“Self-rehabilitation”, “Correction”, “Healthy + BAS “FLP-MD”“ groups) and the control indicators (“Control” group); ### $P < 0.001$, the difference is significant between the values of the indicator in the “Correction” and “Self-rehabilitation” groups

Source: developed by the author of this study

In case of administration of BAS “FLP-MD” in animals with experimental fatty hepatosis, in all three bile samples the concentration of bilirubin sulphate did not differ from the control and, respectively, was 39.3%, 36.0%, and 36.5% higher than the values of these indicators in the “Self-rehabilitation” group (Table 4). Therewith, there were no changes in the concentration of bilirubin sulphate in the blood samples of experimental animals with modelled hepatopathology and with the use of BAS “FLP-MD” (Table 1). Consequently, no accumulation of this bilirubin metabolite in the inner environment of the organism was observed.

Therewith, the study of the content of bilirubin sulphate in liver tissue showed that in the case of correction of the conditional liver status in animals with modelled fatty hepatosis, the concentration of this metabolite increased by 39.1% compared with the control (Table 2). As a result, it was found that under the influence of the components of the bioadditive in the conditions of modelling hepatopathology, the processes of formation of bilirubin sulphoconjugates by the enzyme systems of hepatocytes intensified, which positively characterises the neutralising function of the liver. D.O. Melnychuk *et al.* (2014) noted a comparable increment in the concentration of bilirubin sulphate in liver tissue (by 33%) after exposure of rats to toxic doses of cadmium chloride (for 14 days). According to the researchers, growing of the content of bilirubin sulphate in rat liver tissue after xenobiotic exposure may indicate an increase in the role of neutral pair compounds formation in case of compensatory intensification of detoxification processes.

Back in the 1990s, H.S. Kaufman *et al.* (1991) showed that bilirubin monoglucuronide in bile interacts with bile acids and is incorporated into mixed micellar bile particles. Thus, bilirubin monoglucuronide displaces cholesterol from bile micelles to cholesterol

vesicles, which contributes to the formation of its monohydrate crystals, which provokes the development of cholelithiasis. This is not the only evidence of the pathogenetic significance of disorders of bilirubin glucuronide metabolism and excretion. Adverse drug reactions may be caused by changes in bilirubin metabolism and the development of jaundice. As noted by X. Yang *et al.* (2022), the leading mechanism appears to be the inhibition of bilirubin glucuronidation mediated by UDP-glucuronosyltransferase 1A1 (UGT1A1) and the factual competition of many compounds, particularly those containing phenolic hydroxyl groups, with the endogenous metabolite bilirubin. As a result, the activity of UDP-glucuronosyltransferase 1A1 towards bilirubin is inhibited and the formation of bilirubin glucuronide is reduced. According to E. Järvinen *et al.* (2022), glucuronic and sulphate metabolites are mostly unable to freely penetrate the plasma membranes of cells. Therefore, for their entry into hepatocytes and excretion into bile, proper activity of organic anion transporters of the basal domains of cell membranes and multidrug-resistant transporters of the apical domains of plasma membranes of liver cells is required. Therefore, changes in the concentration of bilirubin glucuronide in bile may be caused by the effect of hepatotropic factors on the transport systems of the hepatocyte plasma membrane, which ensure the excretion of this form of bilirubin to the bile ducts.

It was found that in animals with modelled fatty hepatosis, the concentration of bilirubin glucuronide in all three bile samples was significantly lower than in control bile samples. Specifically, in the first bile sample of the “Self-rehabilitation” group, the concentration of bilirubin glucuronide decreased by 34.0%, in the second sample – by 35.8%, and in the third sample – by 36.4% compared to the values of this indicator in the control group (Table 5).

Table 5. Bilirubin glucuronide content (mg%)
in bile of experimental rats under experimental conditions ($M \pm m$, $n = 8$)

Experiment time, min	Group of rats		
	“Control”	“Self-rehabilitation”	“Correction”
30	11.69 $\pm$ 1.89	7.72 $\pm$ 1.09***	10.96 $\pm$ 2.18###
60	11.81 $\pm$ 1.81	7.58 $\pm$ 1.27***	11.27 $\pm$ 2.43###
90	11.06 $\pm$ 1.82	7.03 $\pm$.21***	10.52 $\pm$ 1.83###

Notes: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.01$, statistically significant differences between the indicators of the experimental groups (“Self-rehabilitation”, “Correction”, “Healthy + BAS “FLP-MD”“ groups) and the control indicators (“Control” group); ### $P < 0.001$, the difference is significant between the values of the indicator in the “Correction” and “Self-rehabilitation” groups

Source: developed by the author of this study

When comparing the “Self-rehabilitation” and “Correction” groups, it was found that the content of bilirubin glucuronide in animals administered the bioadditive exceeded that of rats with experimental fatty hepatosis. Thus, in the first sample of bile from the “Correction” group, the content of bilirubin glucuronide was 42.0% higher than in the bile from the “Self-rehabilitation” group, in the second sample – by 48.7%, and in the third sample - by 49.6%. Therewith, no statistically significant differences were observed between the bilirubin glucuronide content in the bile samples of the “Control” and “Correction” groups (Table 5). In the blood of animals with artificially reproduced fatty hepatosis, the concentration of bilirubin glucuronide decreased by 44.4% compared to the control values, and in case of administration of the bioadditive to both intact animals and rats with hepatopathology, this indicator was close to the control values (Table 1). In the liver tissue, the content of bilirubin glucuronide in both fatty hepatosis and the use of the BAS “FLP-MD” was substantially lower than in the control (Table 2). However, we believe that the mechanisms of this decline are different.

Namely, in the context of artificial modelling of fatty hepatosis, a reduction in the content of bilirubin glucuronide in liver tissue is associated with a general decrease in bilirubin levels in the body due to the characteristic development of anaemia in this pathology (Gryshchenko et al., 2019). The use of a phospholipid preparation intensifies the transport of bilirubin glucuronide from hepatocytes. Thus, the use of milk phospholipids in the BAS “FLP-MD” in sick rats has a pronounced restorative effect on the processes that determine the formation and flow of bilirubin glucuronide into the bile in animals with experimental fatty hepatosis.

It was found that in patients of the “Self-rehabilitation” group, in all three bile samples, the concentration of the components of the total bilirubin fraction monoglucuronide and monoglucoside was lower than in control samples by 26.5%, 31.2%, and 25.5%, respectively. Whereas oral administration of BAS “FLP-MD” in rats of the “Correction” group eliminated the inhibitory effect on the concentration of the components of the studied total bilirubin fraction monoglucuronide and monoglucoside (Table 6).

Table 6. The content (mg%) of the common fraction of bilirubin monoglucuronide and monoglucoside in the bile of experimental rats under experimental conditions ($M \pm m$, $n = 8$)

Experiment time, min	Group of rats		
	“Control”	“Self-rehabilitation”	“Correction”
30	1.17 $\pm$ 0.15	0.86 $\pm$ 0.18***	1.01 $\pm$ 0.26
60	1.26 $\pm$ 0.24	0.88 $\pm$ 0.12***	1.02 $\pm$ 0.27

Table 6. Continued

Experiment time, min	Group of rats		
	“Control”	“Self-rehabilitation”	“Correction”
90	1.10 ± 0.14	0.82 ± 0.11***	0.94 ± 0.26

Notes: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.01$, statistically significant differences between the indicators of the experimental groups (“Self-rehabilitation”, “Correction”, “Healthy + BAS “FLP-MD”“ groups) and the control indicators (“Control” group); # $P < 0.05$, the difference is significant between the values of the indicators in the “Correction” and “Self-rehabilitation” groups
Source: developed by the author of this study

Thus, according to the described results of determining the effectiveness of the milk phospholipid complex in the BAS “FLP-MD” on the background of simulated liver pathology, it was noted that the endogenous formation and stimulation of bilirubin monoglucuronide and monoglucoside in bile were enhanced. This effect of the BAS “FLP-MD” was manifested in the fact that the content of bilirubin monoglucuronide and monoglucoside in the bile of these animals approached the level of control values. In the blood of animals with fatty hepatosis, a 78.9% decrease in the concentration of bilirubin monoglucuronide and monoglucoside was observed (Table 1), which is possible with the simultaneous development of anaemia in diseased animals, as described earlier (Gryshchen-

ko *et al.*, 2019). This leads to a decrease in the content of heme transformation derivatives in the blood in response to artificial modelling of liver pathology. Administration of the bioadditive to sick animals completely eliminates this depressive effect.

Under the conditions of artificial modelling of hepatopathology, due to the introduction of toxic doses of tetracycline hydrochloride (“Self-rehabilitation” group), the concentration of bilirubin diglucuronide in the bile of diseased animals decreased. Thus, in the first sample of bile from the “Self-rehabilitation” group, the content of bilirubin diglucuronide was 24.0% lower than in the control group, in the second sample – by 22.4%, and in the third sample – by 24.0% (Table 7).

Table 7. Bilirubin diglucuronide content (mg%) in bile of experimental rats under experimental conditions ($M \pm m$, $n = 8$)

Experiment time, min	Group of rats		
	“Control”	“Self-rehabilitation”	“Correction”
30	21.23 ± 2.37	16.13 ± 1.92***	19.90 ± 2.93
60	21.03 ± 1.86	16.31 ± 1.45***	19.89 ± 2.74
90	19.81 ± 1.98	15.06 ± 1.52***	19.44 ± 2.46

Notes: *** $P < 0.01$ statistically significant differences between the indicators of the experimental groups (“Self-rehabilitation”, “Correction”, “Healthy + BAS “FLP-MD” groups) and the control indicators (“Control” group); ### $P < 0.001$, ## $P < 0.01$, the difference is significant between the values of the indicator in the “Correction” and “Self-rehabilitation” groups
Source: developed by the author of this study

Correction of the condition of animals with experimental fatty hepatosis with the help of BAS “FLP-MD” contributed to the restoration of bilirubin diglucuronide content in rat bile. Notably, both in the modelling of fatty

hepatosis and in the group of animals treated with milk phospholipids as part of the BAS “FLP-MD”, no significant changes in the concentration of bilirubin diglucuronide in the blood were detected (Table 1). Therewith, a

19.9% decrease in the concentration of bilirubin diglucuronide was recorded in the liver tissue of the diseased rats, which is probably due to the inhibition of hepatocyte enzyme systems of bilirubin glucuronidation. In the liver samples of animals treated with the BAS “FLP-MD” on the background of experimental fatty hepatosis, the content of bilirubin diglucuronide was 37.0% lower than the control values, which may be due to the intensive formation and excretion of this bilirubin metabolite into the bile ducts.

Hyperbilirubinaemia has always been noted as a typical sign of liver damage of various aetiologies and was considered a marker of the relevant severity of hepatopathology. To a large extent, the accumulation of bilirubin and its metabolites is caused by the activation of certain genetic transcription factors. S. Miura & A. Suzuki (2020) noted that bilirubin accumulation in blood and tissues is associated with over-expression of the transcription factor snail, which leads to impaired secretion of primary bile by hepatocytes and is further manifested by the development of steatohepatitis. Lipid accumulation in liver cells is caused by an excess of snail in steatohepatitis. Eventually, this causes pathological changes in the liver tissue, accompanied by a violation of bile acid homeostasis, the development of further mitochondrial dysfunctions and oxidative stress. It is important that snail induces epithelial-mesenchymal degeneration with subsequent transition to hepatocellular carcinoma. Thus, the overactivity of the above-mentioned transcription factor in knockout and mutant mice with the corresponding gene alleles correlates with fatty liver. Therefore, it is important to find all possible factors that would allow controlling the proper activity of snail in hepatocytes.

In a study of the correlations between total blood bilirubin and the severity of steatosis in patients with fatty liver, X. Ma *et al.* (2022)

pointed out how excess snail may influence the complex relationship between bilirubin levels in the body's internal environment and the intensity of fatty liver. According to their findings, there is a negative correlation between the level of circulating bilirubin in the blood and the risk of developing fatty liver disease. Patients with fatty liver usually have low levels of direct bilirubin in their blood. Therewith, the level of uridine 5'-diphospho-glucuronosyltransferase (UGT1A1) activity positively correlated with the intensity of hepatocyte lipidosis. X. Ma *et al.* (2022) emphasised the need for further research to identify the relationship between fatty liver disease, circulating total bilirubin and UGT1A1 activity. According to the analysis of X. Shu *et al.* (2022), the leading risk factors for the development of fatty hepatosis include obesity, dyslipidaemia, diabetes mellitus with insulin resistance, and metabolic syndrome. Although the aetiology of fatty hepatosis is insufficiently understood, oxidative stress is considered a component of possible pathogenic mechanisms. X. Shu *et al.* (2022) emphasised that simultaneous changes in both direct and total bilirubin are unlikely to be associated with the risk of initial development of fatty liver. However, the risk of more severe fatty liver is inversely related to the level of bilirubin in the blood due to the inability to exercise its antioxidant properties in case of a decrease in its concentration in the body.

Thus, the decrease in the content of various bilirubin metabolites in tetracycline-induced fatty liver disease found in the present study indicates the possibility of a decrease in the body's adaptive capacity in the conditions of this type of liver damage with intensifying pathological changes in all physiological systems. Therewith, modelling of tetracycline-induced fatty liver in *Wistar* rats revealed characteristic changes in bile pigment metabolism

caused by the direct cytotoxic effect of high doses of the antibiotic on hepatocytes. According to the findings of chromatographic analysis of the content of bilirubin and its derivatives in the blood, liver, and bile of rats under artificial modelling of hepatopathology, substantial disorders of bile pigment metabolism were found, which is primarily associated with the level of structural and functional destabilisation of hepatocytes, specifically, their membrane systems. Experimental reproduction of this hepatopathology in laboratory rats helped to determine the corrective effectiveness of milk phospholipids in the composition of the BAS "FLP-MD", the use of which substantially improved the bile-forming and biliary functions of the liver and accelerated the recovery of pigment metabolism in diseased animals.

Conclusions

In conditions of tetracycline-induced fatty hepatosis in male *Wistar* rats, a significant decrease in blood levels of unconjugated bilirubin by 39.3%, bilirubin glucuronide by 44.4%, and the total fraction of bilirubin monoglucuronide and monoglycoside by 78.9% was observed compared to the corresponding control. The use of the milk phospholipid complex in the BAS "FLP-MD" in sick animals eliminated the above effects of pathological changes in the functioning of liver cells and the level of the studied unconjugated and conjugated forms of bilirubin in the blood. The ability of the BAS "FLP-MD" to stabilise the concentration of bilirubin and its derivatives in the blood at a physiological level in the development of experimental fatty hepatosis is one of the mechanisms of its hepatoprotective effect mediated by the reparative, antioxidant, and choleretic properties of milk phospholipids.

Therewith, in the liver tissue of rats with experimental fatty hepatosis, the content of bilirubin glucuronide decreased by 20.7% and

bilirubin diglucuronide by 19.9% compared to the control. The use of phospholipid-containing BAS in rats with experimental fatty hepatosis led to a decrease in the content of bilirubin sulphate, bilirubin glucuronide in the liver tissue, bilirubin monoglucuronide, and the total fraction of monoglucoside and bilirubin diglucuronide, which is associated with the intensive excretion of conjugated forms of bilirubin into the bile ducts, and thus the choleretic effect of milk phospholipids. The accumulation of unconjugated bilirubin in the liver tissue of rats with the use of the BAS "FLP-MD" against the background of fatty hepatosis indicates the intensification of its removal from the blood by the transport systems of hepatocytes under the influence of the active substances of the bioadditive. The ability of the BAS "FLP-MD" to enhance the movement of unconjugated bilirubin from the blood to liver cells under conditions of modelled fatty hepatosis is another mechanism of its therapeutic effect, which prevents the development of intoxication and jaundice.

The revealed unidirectional changes – a decrease in the concentration of unconjugated bilirubin and its conjugated forms in bile both in tetracycline-induced fatty hepatosis and in the conditions of administration of BAS "FLP-MD" to sick rats – are conditioned by different mechanisms. Specifically, under conditions of experimental fatty hepatosis caused by inhibition of protein synthesis processes and associated anaemia, a decrease in the pigment content in bile was observed. We assume the fact that under the action of the BAS "FLP-MD", synthetic and energy-dependent transport and enzymatic processes of bilirubin biotransformation are enhanced, which, together with the simultaneous choleretic effect of the investigated phospholipid bioadditive, contributed to a decrease in the concentration of unconjugated and conjugated bilirubin in bile. The

established regularities allow recommending this bioadditive as a means of corrective therapy for the development of pigment metabolism disorders in animals with fatty hepatosis, as well as for the prevention of cholestasis and bilirubin encephalopathy.

In further experimental studies, it is planned to study the features of protein biosynthesis

in the liver tissue of the liver in simulated toxic form of fatty hepatosis in animals.

None.

None.

Acknowledgements

Conflict of Interest

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Похідні білірубіну в жовчі, крові та печінці щурів за корекції експериментального жирового гепатозу

Йозеф Іллек

Доктор ветеринарних наук, професор
Університет ветеринарних наук Брно
61242, вул. Палацького, 1946/1, м. Брно, Чеська Республіка
<https://orcid.org/0000-0002-1374-7918>

Станіслав Весельський

Доктор біологічних наук, старший науковий співробітник
Київський національний університет імені Тараса Шевченка
01601, вул. Володимирська, 64/13, м. Київ, Україна
<https://orcid.org/0000-0001-9971-0333>

Андрій Потоцький

Аспірант
Національний університет біоресурсів і природокористування України
03041, вул. Героїв Оборони, 15, м. Київ, Україна
<https://orcid.org/0000-0003-2500-6647>

Вікторія Грищенко

Доктор ветеринарних наук, професор
Національний університет біоресурсів і природокористування України
03041, вул. Героїв Оборони, 15, м. Київ, Україна
<https://orcid.org/0000-0001-6601-1392>

Євдокія Решетнік

Кандидат біологічних наук
Київський національний університет імені Тараса Шевченка
01601, вул. Володимирська, 64/13, м. Київ, Україна
<https://orcid.org/0000-0003-1084-842X>

Анотація. Актуальність дослідження полягає у відсутності на сьогодні достовірної інформації щодо провідних аспектів молекулярних механізмів виникнення поширеного в ссавців жирового гепатозу та його небезпечних ускладнень – фіброзу, цирозу та гепатоцелюлярної карциноми. Ці обставини істотно ускладнюють своєчасну діагностику зазначеної гепатопатології та знижують ефективність лікування. Унікальним і властивим для печінки є метаболізм похідного геміу – білірубіну, який завдяки ензимним системам гепатоцитів зазнає трансформації з утворенням нейтральних сполук – глюкуронокон'югатів та сульфокон'югатів, що зазнає змін і потребує аналізу за жирового гепатозу в тварин. Мета дослідження полягала у визначенні характерних змін вмісту некон'югованого білірубіну та його сульфокон'югату, глюкуроніду, моноглюкуроніду, моноглюкозиду і диглюкуроніду в крові, жовчі та печінці щурів за моделювання жирового гепатозу й призначення відновлювальної терапії. Для цього використовували метод тонкошарової хроматографії. Встановлено, що за тетрацикліндукованого жирового гепатозу в лабораторних щурів відбувалося зменшення в крові концентрації некон'югованого білірубіну на 39,3 %, білірубіну

глюкуроніду на 44,4 %, сумарної фракції білірубіну моноглюкуроніду та моноглікозиду на 78,9 %. Пероральне введення хворим тваринам комплексу фосфоліпідів молока у складі біодобавки «FLP-MD» забезпечувало підтримання вказаних показників на рівні контролю. У тканині печінки хворих щурів знижувався вміст білірубіну глюкуроніду й білірубіну диглюкуроніду. У разі застосування цим тваринам фосфоліпидовмісної біодобавки в тканині печінки зростає рівень некон'югованого білірубіну на 22,2 %, але зменшувався вміст його кон'югованих форм. У жовчі хворих щурів за самореабілітації зменшувався вміст усіх досліджуваних фракцій білірубіну. Компоненти біодобавки «FLP-MD» за жирового гепатозу в щурів не впливали на зменшення вмісту некон'югованого білірубіну в жовчі, але усували інгібуючий ефект змодельованої патології на вміст усіх кон'югованих форм білірубіну. Отримані результати можуть бути використані в якості біохімічних маркерів для контролю за показниками пігментного обміну за розвитку жирового гепатозу в ссавців

Ключові слова: жовчні пігменти; фосфоліпіди молока; тетрацикліну гідрохлорид; тонкошарова хроматографія; коригувальна терапія; жирова дистрофія



2024. Volume 15, No. 2. P. 66-101

Received: 25.01.2024 Revised: 17.04.2024 Accepted: 29.05.2024

DOI: 10.31548/veterinary2.2024.66

UDC 636.09:57.017.6:616-091.1:001-043.95

Thanatognomonic substantiation of the forensic veterinary diagnosis in the death of dogs and cats due to haemodynamic disorders and hypoxaemia

Roman Kazantsev*

Postgraduate Student

State Biotechnological University

62341, 1 Akademichna Str., v. Mala Danylivka, Ukraine

<https://orcid.org/0000-0002-4479-1516>

Ivan Yatsenko

Doctor of Veterinary Sciences, Professor

State Biotechnological University

62341, 1 Akademichna Str., v. Mala Danylivka, Ukraine

<https://orcid.org/0000-0001-8903-2129>

Abstract. At present, there are no legally consolidated, regulated procedures for conducting forensic veterinary examination of animal cadavers in Ukraine, which makes it difficult for law enforcement agencies to objectively qualify forms of administrative and criminal offences within the framework of pre-trial investigations. Therefore, scientifically sound research results in the field of animal treatment are of particular relevance. The purpose of this study was to substantiate the cause-and-effect relationships in the genesis of violent death of animals caused by mechanical injuries, thermal trauma, and mechanical asphyxiation with the identification of thanatognomonic patterns according to a two-level system of evidence. Methods of comprehensive forensic veterinary examination of animal cadavers were employed, followed by logical and epistemological processing of the results. It was argued that the main links of thanatogenesis in the case of death of animals from these species are irreversible haemodynamic disorders and acute hypoxemia. Based on this approach, an estimate of the area of pathological damage to internal organs incompatible with life is proposed, followed by an “expert hypothesis” regarding the determining cause of the animal’s death. The morphological positions of the forensic veterinary diagnosis in the form of thanatognomonic positions inherent in a certain subspecies of violent animal death were distinguished by two levels

Suggested Citation:

Kazantsev, R., & Yatsenko, I. (2024). Thanatognomonic substantiation of the forensic veterinary diagnosis in the death of dogs and cats due to haemodynamic disorders and hypoxaemia. *Ukrainian Journal of Veterinary Sciences*, 15(2), 66-101. doi: 10.31548/veterinary2.2024.66.

*Corresponding author



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of evidence and their criterion informativeness was proved. Patterns of rapid death of animals of the first level of evidence common to the fatal consequences of mechanical and thermal trauma and mechanical asphyxiation were established: “asphyxiated heart”, congestive venous hyperaemia of internal organs, Tardieu’s spots, alveolar emphysema of the lungs. Patterns of the second level of evidence were selectively randomised and their species specificity was presented. The circumstances under which animals suffer fatal mechanical and thermal injuries and asphyxiation were detailed, and the mechanisms of death were substantiated. The study focused on the specific features of argumentation in concluding the necessary direct causal link between the genesis of violent death and the cause of death. The findings obtained following the standard of evidentiality “study of the object of forensic veterinary examination at different morphological levels” are recommended for implementation in the practice of forensic veterinary examination of animal cadavers

Keywords: bodily injury; thermal trauma; mechanical asphyxia; animal thanatogenesis; argumentation of an expert opinion

Introduction

In Ukraine, the national legislation regulates the issues of organising and conducting forensic examinations in a fairly comprehensive manner. However, the procedures for conducting a forensic veterinary examination of animal cadavers are currently not legally consolidated. Therefore, as reported by A. Yashchenko (2022), the development of concrete issues of forensic veterinary examination is essential to assist law enforcement agencies in the correct qualification of forms of animal abuse offences during pre-trial investigation, trial of crimes against animal health, and imposition of sanctions for grave consequences. To eliminate the gaps in this area of expertise, the study analysed the possibilities of forensic veterinary examination as a new type of forensic examination, its current state and prospects for development in Ukraine, and forensic cases of examination of animal cadavers with signs of violent death from mechanical injuries, asphyxiation and overall cruel treatment. Therewith, M. Yushkov (2022) focuses on the prevention of administrative offences in the field of animal treatment: an individual who keeps an animal is obliged by law to ensure

the prompt provision of veterinary services, including examination and treatment, to immediately notify state medical or veterinary institutions of cases of injury to humans or other animals by an animal and, in this case, to immediately bring it to a veterinary medical institution for examination.

If the investigating authorities establish objective circumstances that, individually or in combination, give grounds to believe that the cause of the animal’s death was bodily injury or mechanical asphyxiation, a forensic veterinary examination is ordered. The purpose of the latter is to establish the initial (determinative) cause of death and the final forensic veterinary diagnosis based on a comprehensive examination of the case file and objects of examination.

J.K. Serdioucov & D.Yu. Shkundia (2021) found that in the practice of forensic veterinary examination, there are often cases of statutory forms of animal abuse: killing animals with firearms or bladed weapons, sharp instruments or blunt objects, as a result of falling from a height, drowning, death in road accidents, etc. Among the most popular methods for determining the age of death of animals, the

researchers cite the following: visual-palpatory, thermometry, morphometric, and morphological (including histochemical, histological, and micromorphometric examination), biophysical (including the adhesive method, measurement of the water-binding capacity of tissues), and a wide range of biochemical methods aimed at determining the content of certain chemicals – products of decay and breakdown of tissues – in the tissues and fluids of an animal cadaver.

Based on the specific features of the pre-trial investigation and the principles of legal proceedings, during which only a direct link between the actions of a particular person and the consequences that have occurred is accepted as evidence of an offence, one of the difficult tasks of forensic veterinary examination is to establish a causal link between animal trauma and adverse outcomes. After all, the objective side of the crime regulates the need to establish a causal link between the actions of a particular person and the consequences that have occurred.

Establishing the cause of death during the forensic veterinary examination of an animal cadaver is important, and, in the context of forensic veterinary examination, the cause of death of an animal should be interpreted in the same way by forensic experts and law enforcement agencies. The conclusion as to the cause of death (accident or violent injury) is the prerogative of law enforcement agencies, but in some cases, a forensic veterinarian may point to signs inherent in a particular type of death. Therewith, law enforcement agencies mainly identify direct causal links between the unlawful act and its consequences, which is also related to the position of the customers of the expertise. At the same time, the direct link between cause and effect must be clear to all parties. The latter, in contrast to a possible connection, is characterised in all cases by the constant occurrence of consequences with other qualitative characteristics.

Therefore, if the forensic veterinary examination of the cadaver does not establish a direct causal link between the injury and the condition that directly caused the death of the animal, law enforcement agencies will have no grounds to establish a direct causal link between the violent actions of the person who caused the injury and the death of the animal.

As practice shows, professional mistakes by veterinary specialists performing diagnostic autopsies of animal cadavers occur precisely during their detailed description, which is arguably caused by the lack of an accepted unified algorithm. In this context, V. Delgado *et al.* (2021) and R. Munro (2022) believe that for the scientific solution of these problems, it is necessary to continue the search and substantiation of standardised algorithms and criteria in the forensic veterinary examination of animal cadavers.

The purpose of this study was to substantiate the cause and effect relationships in the genesis of violent death of animals due to haemodynamic disorders and hypoxemia based on the findings of original research based on the proposed approach with the identification of thanatognomonic patterns (the definition “thanatognomonic pattern” should be understood as specific signs inherent in a particular type of violent death of animals), which will increase the level of reasonableness of the conclusion of a forensic veterinary expert.

Literature Review

N.M.A. Parry & A. Stoll (2020) note that various issues in the field of forensic veterinary medicine are constantly discussed in the pages of periodicals. Among the studies of Ukrainian researchers on the problem of establishing causal relationships in animal cadavers in criminal cases, one can distinguish the studies of V. Lemishevskyi (2021), who detailed the case of a forensic veterinary examination of a dog cadaver

with signs of violent death, Yu. Fedyk & I. Besaha (2023), who highlighted the evidentiary value of the veterinary medicine specialist's opinion during legal proceedings, I. Yatsenko & R. Kazantsev (2021) showed the features of the structure of the conclusion of a forensic veterinary expert based on the findings of the study of poultry cadavers. In the corpus of currently accumulated data, B. Borysevich *et al.* (2024) summarised information on mechanical asphyxia in small animals, while M. Skrypka *et al.* (2023) established the pathomorphogenesis of mechanical injuries of the axial skeleton of animals in case of fatal polytrauma.

Analysing systematic reviews by foreign researchers published in recent years, there is some limited information on the use of algorithms, and those that are used are mainly represented by literature on forensic medicine, which does not specify the range of issues addressed by forensic veterinary examination. Clearly, this is associated with a certain conservatism in the argumentation and formulation of the forensic veterinary diagnosis for certain subtypes of violent death of dogs and cats. Among the publications of foreign researchers in this context, it is worth highlighting the systematic review by N. Kulnides & A. Lorsirigool (2023), which outlines general approaches to the forensic veterinary examination of animal cadavers affected by cruelty. All these researchers argued for fatal consequences mainly in the context of certain types of violent animal deaths and emphasised the need to use professional knowledge in forensic veterinary examination, while S. Monsalve *et al.* (2021) in a separate report stressed the need to improve the theoretical and practical training of specialists in forensic veterinary examination.

Notwithstanding the above, the reasoning behind the conclusion of a forensic veterinary expert in criminal proceedings for animal

deaths caused by acute haemodynamic disorders and hypoxia has not yet received proper coverage. Specifically, the main links in the thanatogenesis of mechanical, thermal trauma, and certain subtypes of mechanical asphyxiation are not generalised, since the establishment of the mechanism of death and the necessary causal link between the injuries and the cause of death of an animal substantially affects the outcome of the pre-trial investigation of criminal offences.

Considering the above, there is every reason to state the existence of a significant empirical body of work, which should form the theoretical framework for further scientific research towards solving fundamental and applied problems of forensic veterinary medicine.

In summary, one of the key tasks of forensic veterinary examination is to argue the necessary causal link between the lifetime injuries of an animal and its death, which is also emphasised by D. Doukas (2022). The development of new methods, techniques, and means of forensic veterinary examination of animal cadavers in various types of violent death and their implementation in expert practice can positively affect the efficiency and effectiveness of forensic veterinary examination, providing a reliable, substantiated, and objective expert opinion in a categorical form, which directly affects its assessment as evidence and, accordingly, the court decision.

Materials and Methods

This study is a fragment of the research work of the National Scientific Centre "Hon. Prof. M.S. Bokarius Forensic Science Institute", Kharkiv (state registration number 0121U100299). The methods of extrapolation and logic were applied retrospectively based on the results of the analysis of 300 veterinary medical opinions during 2013-2023. In each sectional case, the study of experimental

animal cadavers was performed following the methodology approved by the Ministry of Justice of Ukraine (Yatsenko & Kazantsev, 2023).

The methods employed were those of a modern clinical trial standard: objects, patterns, inclusion, and exclusion categories, statistical sampling, representativeness, randomisation, levels of the pyramid of evidentiality. The objects of the study were selected by random sampling – examinee dogs and cats and examinee dog and cat cadavers (170 dogs and 150 cats aged from 1 day to 10 years), forensic veterinary experts' opinions. Inclusion criteria: mechanical injuries, thermal injuries, and mechanical asphyxia in dogs and cats with *exitus letalis*. The exclusion criteria were non-fatal mechanical injuries, thermal injuries, and mechanical asphyxiation of dogs and cats.

A randomised prospective study was conducted in the inpatient setting of veterinary medical institutions in Kharkiv (2021-2023; cats, n = 100; dogs, n = 100) by clinical observation of the posttraumatic period for seven days. All animal manipulations were carried out in strict compliance with the principles of bioethics regulated by the Procedure for Conducting Experiments and Experiments on Animals by Scientific Institutions following the Law of Ukraine No. 3447-IV (2006). In case of death of dogs and cats, their cadavers were autopsied no earlier than two hours postmortem. According to the Law of Ukraine No. 287-VIII (2015), the cadavers of all animals were classified as Category III by-products.

For histomorphological studies of the loci of pathological lesions and/or injuries, cylindrical necropsies were taken from them during *sectio* using Panch (China), then histological preparations were made from them according to the generally accepted method (Horalskyi *et al.*, 2015) and then stained with haematoxylin and eosin. The obtained histotopograms were evaluated in the field of view of a 10×40 optical

microscope “Granum R50” (China). The most representative changes were photographed using a TouPCam UCMOS03100KPA camera (China) integrated with a microscope and analysed following the recommendations (Raskin *et al.*, 2022).

Macro photographs of dog and cat cadavers were taken with an Olympus E-450 SLR camera (Japan). Digital radiographic images were obtained using the X-ray Device PCMAX-40HP (Korea), magnetic resonance imaging – ECHELON Smart 1.5T (Japan), X-ray tomography – 32GE Optima CT 540 (Netherlands), and endoscopy – Olympus CLV-160 (Japan).

Photographic screens obtained during radiological and endoscopic examinations of forensic veterinary objects were detailed using the RadiAnt DICOM Viewer 2023.1 software package. All photos were processed in Photo Frame Studio 3.0 on a personal computer. The photogrammetry was performed using a forensic ruler designed for large-scale photographic recording of objects. The statistical analysis of the original data was performed by calculating the percentage of expert cases.

Results and Discussion

When analysing the forensic veterinary case files, it was noted that the tasks to be solved by the investigating authorities vary depending on the case. However, questions about the cause of death are always encountered in the examination of an animal cadaver.

Our expert practice shows that each traumatic case is a consequence of a concrete cause, while the pathomorphogenesis of a traumatic disease materially reflects the cause of the animal's injury. Death caused by the adverse effects of a traumatic animal disease is the result of a sequential chain of concrete cause-and-effect relationships. However, it is important to emphasise that this sequence requires a sufficient set of conditions that further determine

the development of the consequences. Thus, only a comprehensive determination of the pathomorphogenesis of the injury can confirm or refute the existence of a causal relationship between a particular event and the death of an injured animal.

Authors argue that the forensic veterinary diagnosis, as the quintessence of an autopsy with the statement of changes that have arisen as a result of the impact of certain damaging factors from the beginning of their action to the fatal outcome, is based only on this principle, and allows clearly tracing the links between the root cause of death, its fatal complication, and the immediate cause of death. To establish a forensic veterinary diagnosis, it is first of all advisable to identify the underlying disease (the initial cause of death), which in a monocausal diagnosis is represented by one nosological unit, and in a bicausal diagnosis – by two (competing, combined, or primary and background diseases). The main nosological entity that includes the fatal complication (the immediate cause of death) should be given as the second item of the diagnosis. Background conditions (if any) that aggravate the underlying cause should be outlined at the end. We believe that unfavourable complications of the underlying cause of death in the form of morphological positions should be considered as natural manifestations of the underlying cause of death, combined by a common thanatogenetic mechanism. At the same time, the immediate cause of the animal's death with the corresponding manifestations as a result of an independently completed pathogenesis becomes the basis for the development of the dying process and leads to a premorbid state.

Authors state that the forensic veterinary diagnosis should be based on a set of objective evidence obtained during a comprehensive, thorough examination of the animal cadaver, even in cases of seemingly obvious direct cause

of death, including morphological positions, and must be supplemented by the results of microstructural tissue analysis. For instance, in the case of acute blood loss syndrome, we note morphological changes in the lung parenchyma, which are equally likely to characterise both haemorrhagic shock and congestive venous hyperaemia, depending on the duration of the terminal period, circulating blood volume deficit, etc. Thus, to determine the mechanism of death in this condition in each case, it is necessary to conduct additional verification of the locus of the lesion at different levels of structural organisation.

During the forensic veterinary autopsies of the examinee cadavers of animals whose death occurred rapidly from mechanical injuries or mechanical asphyxiation, the presence of characteristic common morphological positions was established. Authors propose to refer such non-specific signs to general asphyxial signs (patterns of the first level of evidence) in the diagnostic algorithm for differentiating the direct cause of animal death.

To follow the principle of objectivity in substantiating the conclusion of a forensic veterinary expert, authors consider it necessary to distinguish specific signs of traumatic injury (patterns of the second level of evidence), namely, the presence of body damage, the pleomorphism of the manifestations of which depends on the location and nature of the injury; acute blood loss; general anaemia (background condition in the form of circulatory anaemic hypoxia). It was established that the variants of transformation of cadaveric phenomena largely depend on thanatogenesis, the features of which differ depending on the species and conditions of animal death.

Objectification of the cause-and-effect relationship between severe mechanical trauma of an animal and the onset of death in a hospital of veterinary medicine based on the

analysis of clinical and morphological epicrisis requires a retrospective reproduction of the development of the premortem state and a conclusion on the adequacy of the amount of veterinary care provided. Based on the analysis of case files, clinical and sectional epicrisis reports, and direct clinical observation, we emphasise that most often the adverse effects of traumatic disease in animals occur, specifically, due to causes that can be systematised into two groups. Firstly, this is morphological damage. These include life-threatening injuries: a) gross anatomical destruction (crushing or separation) of the body; b) considerable damage to vital organs (e.g., cardiac tamponade with blood due to extra-mural infarction, brain and/or heart contusion); c) acute bleeding: 1 – external, 2 – internal; d) obstruction of the main vessels by traumatic emboli (air, tissue, etc.); e) mechanical asphyxia: 1 – aspiration of the respiratory tract with blood, 2 – traumatic laryngeal oedema; f) combined polytrauma. The second group include life-threatening functional disorders caused by acute organ failure and/or pain shock. Fatal complications of traumatic animal disease include, specifically, anaerobic/purulent infections and intoxication (infectious toxic shock). An injury that does not cause death on its own can be a condition for the aggravation of another factor that causes an adverse outcome.

As a result of the clinical observations of the course of traumatic animal diseases in the inpatient conditions of veterinary medical institutions, we found that within a week of the post-traumatic period, the death of animals occurs depending on certain causes. Thus, in the first day after the injuries, death was caused primarily by acute systemic disorders, specifically, by a violation of the volume and redistribution of circulating blood. Considering this, a complete diagnosis of such disorders should include an assessment of clinical

data, the nature and morphological features of injuries, and other pathological changes that affect the pathogenesis of traumatic illness. On Days 2-3, the onset of cerebral thanatogenesis in the form of destructive cerebral oedema and pulmonary insufficiency, in the form of pneumonitis and non-cardiogenic pulmonary oedema against the background of acute respiratory distress syndrome was observed. On Days 4-5 of the observation period, the manifestations of the systemic coagulopathy phase in the form of disseminated intravascular coagulation syndrome prevailed. After Day 6 of traumatic illness, death occurred with the development of septic conditions, which suggests a prognostically unfavourable outcome of the traumatic injury.

Authors argue that a direct, necessary causal link should be substantiated in all successive links of the (patho)thanatogenesis of traumatic disease. However, it is not possible to establish at what point during a traumatic illness the triggering thanatogenic link is implemented, since aspects of thanatogenesis (its types and mechanisms) differ substantially depending on the concrete type of trauma. This circumstance clearly leads to a high probability of expert error in determining the cause of death and aggravating factors, especially in the case of fatal complications. Undoubtedly, the transition of the pathogenesis of a traumatic animal disease to its fatal outcome occurs gradually, but it is also impossible to clearly define the clinical manifestation that reflects such a mechanism, while the thanatological analysis of a particular expert case during comparison is complicated by the lack of common principles of interpretation of the main thanatological principles among veterinary medicine specialists.

In such conditions, it is advisable to focus, firstly, on functional changes that trigger the chain of links in thanatogenesis and, secondly, on morphological patterns that contribute

to the onset of the fatal outcome of traumatic illness. It is recommended to establish the types of thanatogenesis during a forensic veterinary autopsy using a comprehensive morphological approach to the study of internal organs and to assess the organ whose function has decreased to a critical level and made further ontogeny of the animal impossible. Based on this approach, it is necessary to assess the area of pathological changes in internal organs incompatible with life and build an “expert hypothesis” about the cause of death.

Probably, irreversible metabolic disorders associated with oxygen consumption deficiency are still a key link in the thanatogenesis of animals in critical condition and directly cause death in the early post-traumatic period. The systemic inflammatory response that develops after a week is a reflection of the immunological conflict caused by the inability to control the localisation of the infection against the background of consumption coagulopathy, cytopathic hypoxia, which causes mono/multiorgan failure during the development of general inflammation. Endogenous intoxication caused by acute or chronic kidney disease substantially increases the risk of mortality in the long-term post-traumatic period. Therewith, acute kidney damage was diagnosed in 50% of animals admitted to veterinary hospitals with a history of traumatic illness.

The development of acute heart failure plays a crucial role in thanatogenesis after concussions of the corresponding reflexogenic chest area. The most common cause of pulmonary end-stage disease is mechanical asphyxia. The cerebral type of terminal condition is inherent in death occurring in the first hours due to traumatic brain injury, massive blood loss on an intact premorbid background, carbon monoxide poisoning, and mechanical asphyxiation. However, in our practice, we most often had to observe a combined type of thanatogenesis of the terminal state, with predominantly hypoxemic, dysmicrocirculatory, and coagulopathic accents, which often had independent thanatogenetic significance due to the interrelated consequences.

A retrospective analysis of the conclusions of veterinary specialists after diagnostic examinations of animal cadavers revealed quite significant, sometimes contradictory, discrepancies between the results of the examination and synthesis parts, which may be due to a low level of professional competence. However, it is reliably known that the largest number of deceased dogs and cats subjected to sectional examination were those that died suddenly, including outside/within veterinary medical institutions, and without evident signs of violent death, which accounted for 40% of the total number of autopsies (Table 1).

Table 1. Retrospective analysis of the leading causes of death of dogs (n = 150) and cats (n = 150) in 2013-2023 in Kharkiv

Type of animal death	Number of cases		Percentage share, %	
	dogs	cats	dogs	cats
I. Sudden death	60	60	40.0	40.0
II. Mechanical damage (by subtypes):				
1. Effects of a sharp object	15	14	10.0	9.3
2. Effects of firearms	8	7	5.3	4.7
3. Rail-associated injury	2	1	1.3	0.7
4. Motor vehicle associated injury	16	18	10.8	12.0
5. Catatrauma	9	11	6.0	7.3
6. Effects of a blunt object	11	9	7.3	6.0

Table 1. Continued

Type of animal death	Number of cases		Percentage share, %	
	dogs	cats	dogs	cats
III. Thermal injury (by subtypes):				
1. Generalised hypothermia	5	4	3.3	2.7
2. General thermoplegia	5	4	3.3	2.7
IV. Mechanical asphyxia (by subtypes):				
1. Aspiration	8	12	5.3	8.0
2. Strangulation	6	5	4.1	3.3
3. Obstruction	3	2	2.0	1.3
4. Compression	2	3	1.3	2.0

Source: developed by the author of this study

Table 1 shows that the total array of mechanical injuries and mechanical asphyxiation manifestly demonstrates the onset of death in 80 (53.4%) dogs and 82 (54.6%) cats, which together accounts for more than half of all cases studied. However, there was no significant relationship between the distribution of mechanical injuries or asphyxiation in dogs and cats by season. It should also be emphasised that there is no significant difference in the statistical distribution by species and subspecies in the overall structure of dog and cat mortality, expressed in the number of registered cases and their percentage. The analysis of the sub-sample of mechanical injuries indicates that 61 (40.8%) dogs and 60 (40%) cats died from them. As for open mechanical injuries caused by sharp and fire-

arms, such cases were observed in 23 (15.3%) dogs and 21 (14.0%) cats.

In the forensic veterinary practice, we have increasingly encountered cases of animal injuries caused by objects commonly used in everyday life. Thus, monitoring studies of the nosological structure of open mechanical trauma of animals admitted to veterinary medical institutions suggest that such injuries are caused by owners or third persons using various tools. The consequences of exposure to such weapons in dogs and cats are comparable – skin damage in the form of wounds that penetrate deeper than the papillary layer. All cases of overt mechanical injuries of dogs and cats were systematised and listed according to the number of diagnosed cases (Table 2).

Table 2. Retrospective analysis of open mechanical injuries of dogs (n = 170) and cats (n = 150) for 2021-2023 in Kharkiv

Type of animal wounds	Statistical distribution						
	Number of cases				Dynamics by years, units		
	dogs	share, %	cats	share, %	2021	2022	2023
scalped	4	2.4	1	0.7	1	1	3
chopped	5	3.1	4	2.9	3	4	2
stabbed	7	4.2	6	3.7	4	5	4
shot by firearm	10	5.9	10	6.9	3	14	3
crushed	16	9.6	16	10.5	8	14	10
bludgeoned	16	9.7	16	10.7	7	11	14
combined	18	10.5	17	11.3	9	12	14
cut	25	14.5	21	14.1	13	15	18
torn	29	16.8	23	15.6	17	19	16
bitten	40	23.3	36	23.6	23	21	32

Source: developed by the author of this study

According to the obtained numerical data (Table 2), the anthropogenic factor was determined to be aetiological with varying degrees of presence in all types of injuries, except for bite wounds spontaneously induced by other animals. With such injuries, the skin flap had a characteristic bite mark. F. Iarussi *et al.* (2020) proposed to transfer the nosology of injuries caused by dog bite wounds to the objects of forensic veterinary research, since the consequences of such injuries can be not only civil but also criminal in nature. The same opinion was shared by C.L. Hsiou *et al.* (2022), who identified the pleomorphism of bite wounds on cat cadavers. The researchers agree with both theses, because this type of injury, according to the data obtained, significantly prevails over other types of wounds among both dogs and cats.

The statistical distribution of cases of open mechanical injuries in animals suggests that the largest number of cases were lacerations (16.8% among dogs, 15.6% among cats), and the smallest number were scalped wounds (2.4% among dogs, 0.7% among cats). Both types of injuries were caused by soft tissue sprains. Depending on the mechanism of traumatic impact of the weapon, typical injuries were observed. Thus, the formation of cut (14.5% among dogs, 14.1% among cats), stabbed (4.2% among dogs, 3.7% among cats), and chopped (3.1% among dogs, 2.9% among cats) wounds is typical of sharp objects. The morphological features typical of such injuries were established in the form of their smooth edges, smooth walls, limited locus of injury, and the absence of connective tissue membranes in the wound channel. The combined type of wounds (10.5% among dogs and 11.3% among cats) was observed under conditions of combined simultaneous exposure to traumatic factors. Most often, combined injuries were recorded in several anatomical areas of the animal body during transport accidents or falls

from a great height. The formation of firearm wounds (5.9% among dogs and 6.9% among cats) is associated with the impact of high-speed kinetic projectiles, such as bullets, shell fragments, shrapnel, etc. During a collision with a sufficiently large solid surface, animals sustained bruises (9.7% of dogs, 10.7% of cats) and crushed wounds (9.6% of dogs, 10.5% of cats) as a result of compression. The morphological features of these types of wounds in the form of uneven, blood-soaked edges and pronounced connective tissue membranes in the lumen of the wound canal were noted.

In terms of the dynamics of mechanical damage, during 2021-2023, certain types of damage were evenly distributed between years. However, in 2022, there was a rapid increase in cases of gunshot wounds and shrapnel injuries among animals, which is clearly related to the hostilities in the monitored area, as dogs and cats were injured during mass shelling with shells and their fragments. At the same time, it was found that there is no correlation between the time of year and the number of cases of animal injuries.

In terms of the topography of lesions in dogs and cats, the characteristic features of localisation were established. Thus, among dogs, wounds were most often recorded in the pelvic limbs, neck, and torso, while among cats – in the neck, torso, and head. There was also a correlation between the type of wound and its location in animals: bitten and torn wounds were most often observed in the neck and torso, while bruises were mostly observed in the head. Mostly cut and puncture wounds were diagnosed on the animals' limbs, apparently because these areas of the body are the first to come into direct contact with various cutting and piercing objects (glass, nails, etc.) in the soil. However, the localisation of cut and stabbed injuries in other parts of the body could have been caused by cruel treatment. There were also isolated

cases of isolated open injuries of other parts of the animal body, but no regular relationship between their location was established.

In forensic veterinary tanatology, when reconstructing the conditions of injury, one of the key tasks is to establish the fact that the injury was caused by a certain instrument, and not by any other, because the identification of this object substantially assists law enforcement agencies in recreating a reliable picture of the event.

Thus, the presented retrospective analysis of the type, location, and nature of open bodily injuries most often diagnosed among dogs and cats will allow, in the future, to pay special attention to law enforcement agencies to search for traumatic instruments when investigating crimes against animal health. This was also emphasised by E.J. Bartelink *et al.* (2022), who investigated the cadavers of dogs with signs of ante-mortem blunt force trauma. The distribution and severity of injuries, including frac-

tures, were mainly localised to the axial skeleton (skull, thorax, spinal column, pelvis), which is partially consistent with the data obtained from our studies and with documented cases of non-accidental injuries (NAI) among domestic animals.

Using the analysis of the examination sections of the forensic veterinary experts' opinions, a certain similarity in the macromorphological pattern of animal injuries was found, which depended on the aetiological factor. We present an algorithm for formulating a forensic veterinary diagnosis and its substantiation based on the established thanatognomonic patterns of certain subtypes of violent animal death caused by bodily injury, thermal injury, and mechanical asphyxia in typical cases.

During the external examination of the cadaver of cat No. 1, scissors were found sticking out of the right ventral abdomen at an angle of 30° (Fig. 1).



Figure 1. Cadaver of a cat with scissor injuries
Notes: macro photo
Source: developed by the author of this study



Figure 2. Cadaver of a cat with scissor injuries
Notes: photographic screen of radiograph in RL projection (X-ray Device PCMAX-40HP)
Source: developed by the author of this study

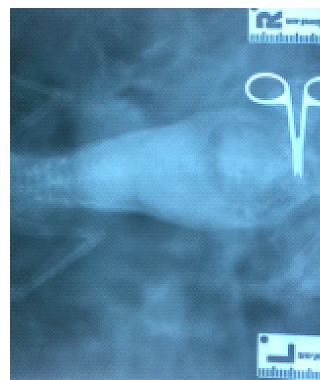


Figure 3. Cadaver of a cat with scissor injuries
Notes: photographic screen of radiograph in DV projection (X-ray Device PCMAX-40HP)
Source: developed by the author of this study

The plain radiographs of the cat's cadaver, performed in RL (Fig. 2) and DV (Fig. 3) projections, show a section of the traumatic

canal located 2.5 cm to the left of the *linea mediana anterior* at the level of the pelvic bones at a distance of 13.7 cm from the hip joint. The

oblique-vertical wound formed on the skin *in situ*, 6.3 cm deep and 0.8 cm long, has smooth, sharply bevelled edges, and is saturated with blood. The subcutaneous base is visible in the lumen of the wound. On the wound walls, numerous dark brown blood particles ranging in size from 0.4×0.6 cm to 0.6×0.8 cm were observed. The wound canal begins on the lateral surface of the trunk, located in the soft tissues from right to left and from top to bottom at an acute angle. The X-ray examination shows no signs of haemorrhage, thus, on the one hand, death probably occurred shortly after the inju-

ry, and on the other hand, the blood saturation of the ribs and wound edges suggests that they were formed *pro vita*. The injuries found on the cat's body caused severe short-term pain before it died. Considering the facts, the initial cause of death of cat No. 1 was a single penetrating stab wound to the abdomen, while the immediate cause of death was traumatic pain shock.

During the external examination of the experimental cadaver of cat No. 2, it was found that a knife with a one-sided blade was sticking out of the area of the 3rd intercostal space of the chest on the left at an angle of 45° (Fig. 4).



Figure 4. Cadaver of a cat with injuries caused by a kitchen knife

Notes: macro photo

Source: developed by the author of this study

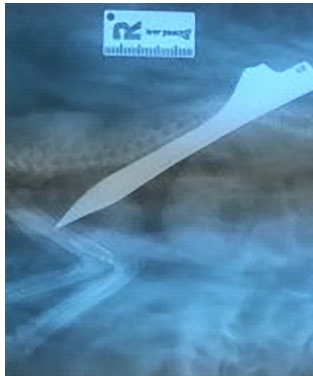


Figure 5. Cadaver of a cat with knife injuries

Notes: photographic screen of X-ray in RL projection (X-ray Device PCMAX-40HP)

Source: developed by the author of this study

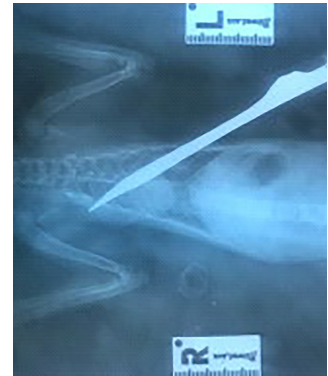


Figure 6. Cadaver of a cat with knife injuries

Notes: photographic screen of X-ray in DV projection (X-ray Device PCMAX-40HP)

Source: developed by the author of this study

Plain radiographs of the cat's cadaver, performed in RL (Fig. 5) and DV (Fig. 6) projections, show a section of the traumatic canal located 5.3 cm to the left of the *linea mediana anterior* at the level of the scapula at a distance of 15.9 cm from the shoulder joint. A 2.0 cm long rectilinear penetrating wound is found on the skin with convergent edges, soaked with blood. The ends of the wound are oriented at 8 and 14 o'clock on the conventional dial, without connective tissue bridges. The wound

edges are clear, the edges are relatively smooth, not incised, the walls are smooth, and bevelled. On the wound walls, numerous dark brown blood particles ranging in size from 0.3×0.5 cm to 0.5×0.7 cm were observed. The subcutaneous base *in situ* revealed a rectilinear wound channel with a depth of 11.3 cm. In the chest cavity, the knife blade touches the heart and penetrates through the lobes of the left and right lungs. The X-ray examination showed no signs of haemorrhage, thus, on the

one hand, death occurred shortly after the injury, while on the other hand, the blood saturation of the ribs and wound edges indicates that they were formed *pro vita*. The injuries found on the cat's body caused severe short-term pain before it died. Considering the above data, the initial cause of death of cat No. 2

was determined to be a single penetrating stab wound to the chest, while the immediate cause of death was traumatic pain shock.

During the external examination of the experimental cadaver of cat No. 3, it was noted that a sewing needle was sticking out of the left eye at an angle of 25° (Fig. 7).



Figure 7. Cadaver of a cat with needle injuries

Notes: macro photo

Source: developed by the author of this study



Figure 8. Cadaver of a cat with needle injuries

Notes: photographic screen of radiograph in RL projection (X-ray Device PCMAX-40HP)

Source: developed by the author of this study

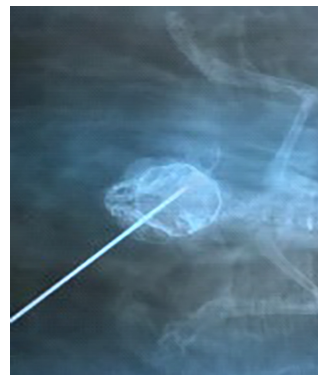


Figure 9. Cadaver of a cat with needle injuries

Notes: photographic screen of radiograph in DV projection (X-ray Device PCMAX-40HP)

Source: developed by the author of this study

The overview radiographs of the cat's cadaver, performed in RL (Fig. 8) and DV (Fig. 9) projections, showed that the needle penetrated the brain substance through the brainstem in an oblique-aboral direction, forming a wound channel. During the enucleation of the injured eye, retrobulbar haemorrhage was detected, and the injury was a rounded wound with a diameter of 0.3 cm, which corresponds to the diameter of a foreign object. The wound canal was straight, its walls were even, crossing the arteries of the arachnoid membrane of the brain and, as a result, bleeding into the subdural space. Due to a puncture wound, minor visual damage was accompanied by a violation of the anatomical integrity of deeply located tissues. Thus, the diagnosis of a stab

wound was difficult, because, depending on the topographical area, such wounds cause health damage of varying severity. Proceeding from the above, it was concluded that the death of the cat submitted for forensic veterinary examination was caused by compression with gross destruction of the brain matter, instantaneously.

During the external examination of the cadavers of dogs with injuries caused by *cutting tools*, typical morphological signs of such injuries were found: their length always exceeds their width and depth, which clearly depends on the sharpness of the blade, the pressure, and the density of the tissues. Due to the pressure of the blade on the skin and underlying tissue while pulling, the tool separates the soft

tissue, causing a laceration. Thus, the incised wound of the experimental dog cadaver in the ventral abdomen (Fig. 10) with entrapment

of the greater omentum was characterised by straight, clear edges with a smooth surface, 23.3 cm long and 12.5 cm wide.



Figure 10. A wound in the umbilical region of the dog's abdomen

Notes: macro photo

Source: developed by the author of this study



Figure 11. Wound in the distal region of the right thoracic limb of a dog cadaver

Notes: macro photo

Source: developed by the author of this study



Figure 12. Wound in the area of the lower leg of the left pelvic limb of a dog cadaver

Notes: macro photo

Source: developed by the author of this study

On the wound walls, numerous rounded dark brown blood particles ranging in size from 0.1×0.1 cm to 0.3×0.3 cm were observed. In this regard, it was concluded that the wound was caused by a cutting object with a sharp blade, as indicated by its somewhat tortuous shape, smooth unabraded edges, relatively flat walls with no signs of active bleeding.

As for the *cutting injury* of the distal thoracic limb (Fig. 11) of the experimental dog cadaver, it occupies an intermediate place between cut and bruised injuries and combines their features in its morphological patterns. The gaping of such a wound in an animal cadaver is visually pronounced, the edges are uneven, there are no signs of external bleeding, but areas of necrosis are visible on the wound ribs and skin flaps. The instrument of injury is a massive yet sharp object with a limited cutting surface. Such an object in this expert case could be a garden shovel. The patterns of wound contracture indicate

that the wound was formed 5-7 days prior, which is explained by the formation of granulation tissue with epithelialisation. Considering the above, there is no direct necessary causal link between the injuries in the form of a cut wound in the ventral abdomen of the examinee dog cadaver and a chopped wound in the distal segment of the right thoracic limb of the examinee dog cadaver and the death of the animals.

Injuries to animals caused by the design features of electric transport tracks (*rail-associated injury*) are rarely recorded, but deserve attention, as the thanatogenesis of this type of violent death is still controversial. The factual injury occurs when the animal's limb hits the railway switch necessary to shift the moving elements of the transport to another railway track (Fig. 12). It is known that such a device consists of frame rails, switch points, and a transfer mechanism. Clearly, the mechanogenesis of this injury is caused, on the one hand, by

compression and overstretching of soft tissues, and, on the other hand, by traumatic amputation of the limb. The specificity of this amputation is probably conditioned by the animal's attempts to free the limb, which was clamped between the rails, while the points clutch. Macromorphological patterns of the pelvic limb fragment of a service dog, separated from the body, submitted for forensic veterinary examination, indicate stripe-shaped skin tears in the form of wide flaps, a wedge-shaped defect of the greater tibia with an overlay of black oily substance in the centre of the bone grind and polished edges with a "metallic sheen". M. Smith-Blackmore & J. D. Bethard (2021), in their report on the analysis of dog bone injuries at different stages of healing, pointed out the expediency of collaborating with forensic anthropologists in resolving the issue of the age of such an injury. It is not possible to answer the question of the cause of death and the lifetime of the injuries in a categorical manner, as the researchers also emphasise. According to our expert hypothesis, the damage to the *v. saphena* is undoubtedly fatal. However, the soft tissues of the stump look somewhat anaemic, while the blood saturation of the edges of the crushed wound is not visually evident. Such haemostasis can be lifelong, provided that skeletal muscle fibres contract reflexively. The researchers believe that the time of fatal outcome is determined by two parameters: a decrease in the volume of circulating blood to a critical level or the time of direct collision with the moving elements of electric vehicles. Thus, in this expert case, the direct necessary causality is two-linked, since the initial cause of death is traumatic amputation at the level of the lower leg, the fatal complication is anaemia of internal organs, and the immediate cause is traumatic pain shock.

Fatal *transport-associated injury* was recorded in 18 (12.1%) dogs and 19 (12.7%) cats

with combined injuries of different parts of the body. In the total number of fatalities caused by blunt objects, the statistical distribution of the number of such cases in dogs and cats is the same. Blunt force injuries (including cat-trauma) were mostly bludgeoned and crushed wounds, which is clearly related to the mechanism of their formation.

Car-associated injury caused by the impact of a large blunt object has always been associated with cranial injury and/or brain contusion in dogs and cats. Damage to certain parts of the animals' bodies was accompanied by the formation of scalp wounds (Fig. 13). Admittedly, rapid death is directly caused by gross anatomical damage to the animal's body that is incompatible with life, such as defragmentation of the head and torso, and damage to vital organs (Fig. 14). Thus, in the case of a combination of gross anatomical destruction of the body of a cat whose death occurred as a result of a complete collision with the wheels of a motor vehicle (Fig. 15), it was not possible to isolate a fatal injury. Therefore, it is necessary to state the death of the animal from the totality of the injuries found, expressed in the following formulation: the cause of death of the cat whose cadaver was submitted for forensic veterinary examination was a combined injury to the head, torso, thoracic, and pelvic limbs caused by a blunt object with a large surface. According to the results of the forensic veterinary examination, a direct and necessary causal link was established between the injuries caused by the pressure of high kinetic energy at high speed on the entire area of the animal and the consequences that occurred in the form of the cat's *exitus letalis*. In this expert case, the causality is one-link, because the initial cause of death was also the immediate cause of death, which occurred due to gross anatomical destruction of body parts incompatible with life.



Figure 13. Scalped wound of the right pelvic limb in a cat cadaver following a car accident

Notes: macro photo

Source: developed by the author of this study



Figure 14. Gross anatomical destruction of the head of a cat cadaver caused by a vehicle collision

Notes: macro photo

Source: developed by the author of this study



Figure 15. Anatomical destruction of the torso of a cat cadaver after being hit by a vehicle

Notes: macro photo

Source: developed by the author of this study

The structure of fatal road traffic injuries to animals has always included severe combined body trauma, which in the vast majority of cases was combined with craniocerebral trauma.

Thus, during the X-ray examination of the experimental cadaver of a dog injured in a road accident as a result of a collision with a moving vehicle, signs of diaphyseal fractures of the long tubular bones of two segments of

the pelvic limbs (thigh and lower leg) with distraction of bone fragments into skeletal muscles were found (Fig. 16). The internal examination of the dog's cadaver revealed injuries inherent in a tangential collision with a vehicle: considerable haemorrhage in the mediastinal organs (Fig. 17), contusion, and focal haemorrhage in the soft tissues of the neck (Fig. 18).

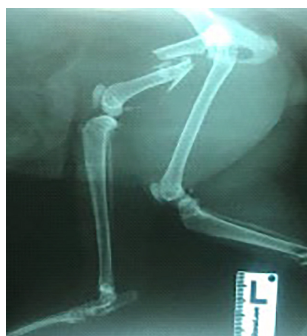


Figure 16. Dog cadaver fragment with diaphyseal fractures of the femur and tibia

Notes: photographic screen of X-ray in LL projection (X-ray Device PCMAX-40HP)

Source: developed by the author of this study



Figure 17. Haemorrhage into the mediastinum of a dog cadaver in case of death from a tangential collision with a motor vehicle

Notes: macro photo

Source: developed by the author of this study

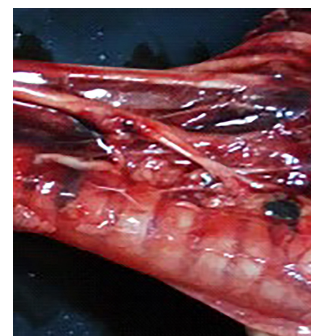


Figure 18. Focal haemorrhages in the soft tissues of the neck of a dog cadaver in case of death from a tangential collision with a motor vehicle

Notes: macro photo

Source: developed by the author of this study

The cause of death of the dog, whose cadaver was submitted for forensic veterinary examination, was a combined trauma in the form of open fractures of the left femur with a ruptured femoral vein, an open fracture of the left tibia, traumatic lung injuries, contusion of the spleen and liver parenchyma with ruptured capsules of these organs, numerous tears of the soft abdominal wall muscle tissue with entrapment of internal organs under the skin of the left lateral abdominal wall. The severe injuries found on the cadaver of the experimental dog were accompanied by the development of traumatic shock and led to its death. We argue that the nature and multifocal localisation of the injuries indicate their formation as a result of a single impact followed by compression with a large surface of a hard blunt object, specifically, a moving vehicle.

Considering the above, the main (initial) cause of death of the animal was a combined trauma with damage to the main vessel, and the immediate cause of death was anaemia of internal organs due to haemorrhagic shock. In this expert case, there is a direct, necessary, single-link causality between the injury sustained by the animal and its death, because the rupture of the main vessel, without any outside veterinary assistance, certainly causes the death of the animal.

In the expert case of a cat *falling from* a nine-storey building, the animal died on the first day of the post-traumatic period in a veterinary hospital. During the external examination of the cadaver's oral cavity, the following injuries were noted: oronasal fissure (Fig. 19), open fracture of the vault bones with violation of the integrity of the dura mater and the anatomical base of the skull (Fig. 20).



Figure 19. Oronasal fissure of a cat cadaver in case of death from catatrauma

Notes: macro photo

Source: developed by the author of this study



Figure 20. Bone structure of the calvaria and skull base of a cat cadaver in case of death from catatrauma

Notes: photo screen of X-ray tomography (32GE Optima CT 540)

Source: developed by the author of this study



Figure 21. Cerebral cavity of a cat cadaver in case of death from catatrauma

Notes: 1 – subdural haemorrhage. Photoscreen of magnetic resonance imaging (ECHELON Smart 1.5T)

Source: developed by the author of this study

Magnetic resonance imaging images of the cadaveric brain indicate a subdural haemorrhage into the cranial cavity (Fig. 21.1), tear of the *articulatio atlantooccipitalis* ligaments, as

evidenced by increased unnatural head mobility and a compression fracture of the antrum. No other injuries were diagnosed during the autopsy. The factual patterns together explain

a certain mechanogenesis of the injuries: trauma to the skull bones with fractures of the calvaria and skull base and tears and haematomas of soft tissues in the corresponding area, which indicates contact of the animal's head with a blunt object with a large surface area or an animal hitting a hard surface (such an object, for instance, may be dense soil or asphalt pavement). All the injuries found on the expert's cadaver were caused during its lifetime, simultaneously, as a result of a catatrauma with a gravitationally accelerated fall velocity, and death occurred instantly. It was found that such a macromorphological picture is typical for catatrauma. In this expert case, the main (initial) cause of death was an open head injury with contusion of the brain matter, while the immediate cause was the destruction of the animal's medulla oblongata due to the distraction of the first cervical vertebrae. When presenting arguments for the cause of death of an animal, attention should also be focused on the presence of a fatal complication – a subdural haematoma. At the same time, V. Lemishevskiy (2021), based on the findings of a sectional study using complete evisceration of the organs of the cadaver, argues that in cases of catatrauma, even with premortem diagnosed craniocerebral contusion of an animal, a strong blow to the chest during a fall from a great height causes bludgeoning of the lungs and heart, which is potentially life-threatening in itself, which must be considered in the presence of competing causes of death. The expert established the following injuries to the cat's cadaver: blunt trauma to the chest with blood imbibition of muscle tissue, haemorrhages in the muscle fascia, ligament rupture, and compression fracture of the transverse rib process of the fifth cervical vertebra. An in-depth analysis of the results of the examination of the cadaver of this animal indicates that the necessary causality between the injuries and the death of the cat is direct, but

two-linked. The initial cause of death and its complications are phenomena that are not tantogenetically related, which is consistent with the data of M. Skrypka *et al.* (2023), who showed methods for identifying and differentially diagnosing spinal injuries and found out the pathomorphogenesis of cervical vertebral distraction in polytrauma in criminal proceedings. In explaining the mechanogenesis of cervical spinal cord injuries caused by sharp traumatic neck flexion, researchers have noted partial or complete ruptures of the intervertebral disc and destruction of vertebral ligaments. It was found that hyperaemia of the vessels of the meninges of the brain was a consequence of blood circulation disorders in the occipital and caudal arteries of the meninges, and mechanical destruction of intervertebral discs or rupture of cervical vertebrae ligaments led to disorders of innervation of various organs and systems of the body and was fatal for the animal. It was proved that distraction trauma of the cervical spine led to oedema and haemorrhage in the caudo-ventral part of the brain substance. A direct correlation between vagus nerve irritation and hyperaemia of large (aorta and pulmonary artery trunk) vessels, caudal mesenteric artery and, accordingly, hyperaemia of the thoracic and abdominal cavities was demonstrated. Thus, the findings obtained by the researchers coincide with observations presented in this study. However, such contradictory information indicates that when addressing the questions of the investigating authorities regarding catatrauma, it is important to determine the landing zone and the impact of the applied force vector.

The trend towards an increase in the number of cases of *firearms-associated injuries* in animals is noteworthy, which undoubtedly reflects certain processes in society and certainly indicates the recent spread of firearms among the population. The number of wound holes in animals caused by this type of weapon in some

expert cases indicates particular cruelty in the treatment of the animal, and the location of the wound holes indicates their chaotic localisation.

Thus, during the external examination of the scalp of a dog (Fig. 22), whose cadaver was



Figure 22. The skin of the left lateral surface of the dog's head with wound holes located in a concentrated manner

Notes: macro photo

Source: developed by the author of this study

The X-ray comparison of the relevant anatomical area proves the presence of more than twenty objects with a cylindrical shape and pyramidal contact surface, X-ray contrast, "metallic" density in the soft tissues of the dog's head (Fig. 23). The X-ray shows that they are topographically localised mainly in the soft tissues of the vault of the skull and, to a lesser extent, in the nasal bones and skull base, in the skeletal muscles of the neck, and correspond to the location of the wound holes on the skin. As for the skin of the pelvic limbs of the experimental dog cadaver (Fig. 24), it also shows numerous wound openings, alike in their macromorphological features to those found during the external examination of the head.

submitted for forensic veterinary examination, numerous injuries, round and oval in shape, with a diameter of 0.5 cm to 0.9 cm, were visually noticeable, while the skin itself was covered with red spots appearing to be blood.

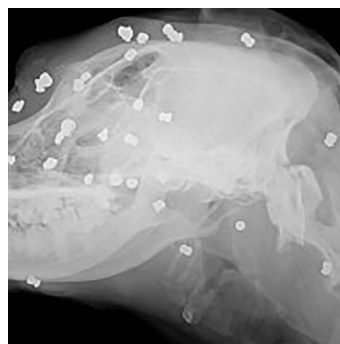


Figure 23. The head of a dog cadaver with objects of "metallic" density

Notes: photographic screen of radiograph in RL projection (X-ray Device PCMAX-40HP)

Source: developed by the author of this study

Analysing the X-ray images of the dog's head and pelvic limbs taken in RL (Fig. 25) and DV (Fig. 26) projections, it was found that all the bullets were located in the soft tissues of the body at different angles, which may indicate shots from different directions from a relatively short distance. Considering this, we agree with the opinion of M. Grela *et al.* (2021) on the expediency of using the radiological method of examining an animal cadaver along with a classical autopsy to assess the fatal consequences of gunshot wounds to dog cadavers. Furthermore, the researchers note that damage caused by gunshots is correlated with the calibre, kinetic energy of the projectile, and its initial velocity, as well as the distance between the muzzle of the gun and the object of the shot.



Figure 24. The skin of the lateral surface of the right pelvic limb of a dog cadaver with rounded wound openings

Notes: macro photo

Source: developed by the author of this study



Figure 25. Fragment of the right pelvic limb of a dog cadaver with objects of “metallic” density

Notes: photographic screen of radiograph in RL projection (X-ray Device PCMAX-40HP)

Source: developed by the author of this study



Figure 26. Fragment of the back of a dog cadaver with objects of “metallic” density

Notes: photographic screen of radiograph in DV projection (X-ray Device PCMAX-40HP)

Source: developed by the author of this study

During the internal examination of the dog's cadaver, it was found that the through soft tissue damage began with a rounded entrance wound with a 0.9 cm diameter soot rim located on the skin in the middle third of the right thigh along its lateral surface (Fig. 27). When a bullet enters soft tissue, it destroys the skin and denser tissue due to its high kinetic energy, forming a rounded hole. It was found that the local defect of the skin of the “minus tissue” type is probably caused by hydrodynamic and contusion effects on the tissue along the trajectory of the traumatic weapon, which is confirmed by our observations. Next, the wound channel is located through the subcutaneous tissue and muscles in the direction from top to bottom, from front to back, and ends with an oval-shaped wound with irregular edges in the distal third of the right thigh along its medial

surface (Fig. 28). Thus, the above bodily injuries have morphological signs of wounds, each of which was separately caused by a shot from a firearm with a compact projectile (bullet) that had a relatively low contusion-dynamic impact on the inner surface of the skin of the dog's cadaver when it left the body.

Explaining the mechanogenesis of the injuries, we argue that the wounds of the right thigh were caused by the penetrating effect of a projectile with high kinetic energy made of metal compounds typical of a jacketed bullet fired in the specified tangential direction. The wounds found are not in the required direct causal relationship with the death of the animal. Considering the above, we believe that even if the pain and suffering experienced by the animal before death did not cause the development of a shock state, the large number

of haemorrhages caused by numerous bullet wounds, together with the systemic changes in the animal's haemodynamics, can be consid-

ered a variant of haemorrhagic syndrome, which may have independent thanatogenetic significance and be the cause of the animal's death.

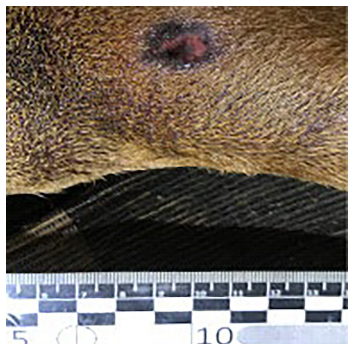


Figure 27. Entrance wound of a dog cadaver with a skin defect of the “minus tissue” type

Notes: macro photo

Source: developed by the author of this study



Figure 28. Exit wound of a dog cadaver

Notes: macro photo

Source: developed by the author of this study

Thus, in the expert practice of mechanical trauma, there is often a combined simultaneous effect of pathogenic factors, which, admittedly, significantly complicates the analysis of further thanatogenesis in each case. It is advisable to point out that despite the diversity of the macromorphological picture of the lethal consequences of mechanical trauma in animals, acute haemodynamic disorders are the basis of thanatogenesis. Such premortem conditions are explained by a decrease in the intensity of hemoperfusion, against which hypovolaemic shock occurs with specific causes in the form of mechanical injuries, specifically, contusion of internal organs with violation of their anatomical integrity, distraction of limbs with soft tissue damage. Notably, in such cases, there was usually considerable blood loss caused by extensive injuries. Haemorrhage plays a triggering role in the thanatogenesis of traumatic shock, as acute blood loss causes an imbalance between the circulating blood volume and the blood vessels. M. Skrypka *et al.* (2024) reached analogous conclusions within the framework

of a pre-trial investigation into cruel treatment that led to the death of an animal (forensic veterinary examination of a dog cadaver with signs of polytrauma). According to the results of the forensic veterinary examination, it was established that the injuries were caused by repeated mechanical interaction (blows) of the left side of the body with a blunt object. The serious injuries included, specifically, disruption of the connection between the bones of the hyoid skeleton and laryngeal cartilage, cerebral haemorrhage, disruption of the integrity of the ligamentous apparatus of the atlanto-occipital joint, fracture of the arch of the first cervical vertebra, mechanical trauma to the subclavian and internal thoracic artery with disruption of their anatomical integrity. Thus, the injuries caused (multiple combined trauma) are in direct causality with the death of the animal. Experts argue that the fatal outcome was the result of hypovolaemic shock caused by violation of the integrity of the subclavian artery and internal thoracic artery by rib fragments. Progressive hypovolaemia clearly reduces

venous pressure in the vessels and provokes the development of transient arrhythmia, which results in a decrease in the minute output of the heart pump. Decompensation of such conditions against the background of acute hypoxemia occurs due to the supra-threshold effect of catabolic products on the vital centres of the animal's medulla oblongata. Excessive exposure to extreme temperatures, according to the data obtained, caused death in 10 (6.6%) dogs and 8 (5.4%) cats.

During forensic veterinary sectional examinations of animal cadavers whose deaths were caused by *general hypothermia*, two permanent non-random macromorphological signs were found in each expert case. Firstly, the dog's non-trivial "compact" body position, which stayed unchanged after death, is noteworthy (Fig. 29). Forensic veterinary autopsy of animal cadavers indicates small-dot haemorrhages in the apical parts of the gastric mucosa folds

(Wischnewsky spots), which are usually linearly sinuous and small in size (up to 0.3×0.5 cm), against the background of general erythema (Fig. 30). Histomorphological examination of the stomach samples of the experimental dog cadaver taken from loci with visually pronounced hyperaemia of the mucous membrane revealed that it was covered with a single-layer foveal epithelium, with changes manifested in the form of desquamation of cylindrical and prismatic cells (Fig. 31.1). Beneath the epithelium is a submucosal layer consisting of loose connective tissue, blood vessels, and dilated glandular excretory ducts. Glands with moderate secretion are located in the lamina propria in the direction from the gastric mucosa to its muscular lamina, which separates the mucosa from the submucosa. The mucosal lamina propria between the glandular ducts and the submucosa are reactive, intensely infiltrated with conglomerates formed by erythrocytes and neutrophils.



Figure 29. Appearance of a dog cadaver in case of death caused by general hypothermia

Notes: macro photo

Source: developed by the author of this study



Figure 30. Diapedetic haemorrhage in the cardiac part of the stomach of a dog cadaver in case of death caused by general hypothermia

Notes: macro photo

Source: developed by the author of this study

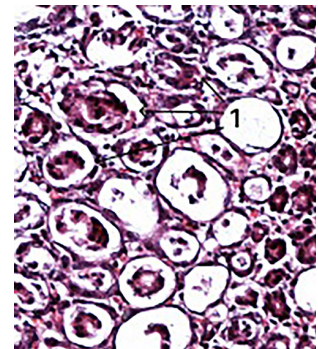


Figure 31. Histotopogram of the gastric mucosa epithelium of a dog cadaver

Notes: 1 – foci of epithelial desquamation with erythrocyte conglomerates. Haematoxylin and eosin, 10×40, (Granum R50 microscope)

Source: developed by the author of this study

Considering the above, we believe that at the onset of general hypothermia, the changes

occurring in the animal's body are hypothetically reversible and limited to systemic

circulatory disorders. In this case, it is crucial to establish the duration of the state of emergency. As a result of the examination, it was established that death from cold injury occurs as a result of vasomotor collapse without icing of the body, and, therefore, the cadaver freezes after the animal's death. Proceeding from this expert hypothesis, the identified patterns of cold injury in animals are always postmortem in nature, conditioning the mechanogenesis of hypothermia. A certain resistance to the effects of low temperature on the animal body necessitates the development of microscopic damage by a sufficiently long exposure to low temperatures. Under such conditions, the animal takes a specific "compact" posture to maintain thermoregulation, followed by the mobilisation of reserve capabilities to maintain homeostasis, specifically, skeletal muscle glycogen

is utilised. However, prolonged exposure to extremely low ambient temperatures is likely to cause a gradual overall decrease in the animal's body temperature against the background of anoxia and anaemia of peripheral tissues, including skeletal muscles, with a characteristic inhibition of the relevant biochemical processes in the body.

Thus, a direct, necessary causal link is logically argued between the established changes that occurred in the cadaver of an animal with a cold injury and its death. In the case of animals whose death was caused by *generalised overheating*, specifically burn injuries, an expert hypothesis about the conditions and cause of death can be built at the stage of external examination of such cadavers. Thus, the burnt hair of the experimental cat cadaver was caused by direct contact with the flame (Fig. 32).



Figure 32. General view of a cat's cadaver in case of death caused by burn injury

Notes: macro photo

Source: developed by the author of this study



Figure 33. Swelling of the brain of a cat cadaver

Notes: macro photo

Source: developed by the author of this study

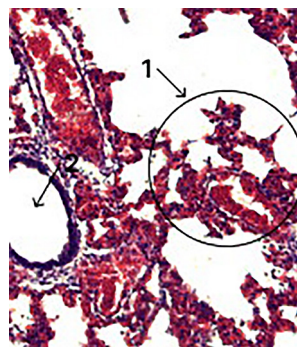


Figure 34. Histotopogram of the lungs of a cat cadaver with alveolar emphysema caused by burn injury

Notes: 1 – destruction of the alveolar septa with numerous diapedeses; 2 – intact alveolus. Haematoxylin and eosin, 10×40, (Granum R50 microscope)

Source: developed by the author of this study

Damage to animal carcasses associated with exposure to, for instance, a burning house, may conceal other changes. Therefore, it is

necessary to verify the vital nature of their formation and substantiate the forensic veterinary diagnosis. The animal's open mouth indicates

terminal tachypnea and the development of respiratory failure caused by inhalation of toxic smoke against the background of hypercapnia and carbon monoxide poisoning. Erythema of the mucous membrane of the oral cavity and the back of the pharynx confirms an acute lesion of the animal's respiratory tract.

The autopsy of the cat's cadaver revealed patterns of cerebral oedema: the furrows of the hemispheres were visually smoothed, while the vessels were markedly blood-filled (Fig. 33), which was explained by hypoperfusion of the nervous tissue and fluid leakage from the narrowed vessels in the interstitium beyond the neurocytes caused by general hypovolaemia. Histomorphological examination of the lungs suggests the development of acute alveolar emphysema, because among the single intact alveoli (Fig. 34.2), numerous lesions of the membranes of the parenchyma with microdiapedeses (Fig. 34.1) were found due to their overstretching by air, which confirms the vital formation of these changes.

Thus, the above signs found in a cadaver with a burn injury, specifically, acute disorders of the haemocirculation of the brain and respiratory tract, are specific to establishing the cause of death of an animal due to general overheating.

In the overall structure of mortality, according to the sub-sample, *asphyxial death* was diagnosed in 19 (12.6%) dogs and 22 (14.6%) cats. Obtained by D. Araújo *et al.* (2021) analogous statistical information. Specifically, according to the researchers, most of the animals that were violently attacked were dogs (87%), outbred (55%), medium-sized (57%), male (58%), and died due to various causes, including asphyxiation (15%). It is known that the asphyxiated state is accompanied by decompensated hypoxia of vital organs, specifically, the brain and heart, which over time leads to the cessation of respiratory excursions due to

central nervous system depletion, transient arrhythmia and, as a result, the onset of animal death.

Authors state that the mechanism of death of animals from mechanical asphyxiation, despite the variety of instruments of trauma, is the same. Based on the mechanogenesis, we consider mechanical asphyxia to be a traumatic injury, with the only difference being that the mechanical factor affects the locus of injury throughout the entire period of death, thereby constantly irritating the corresponding receptors of the reflexogenic zone and exacerbating the pathological manifestations of hypoxia in the animal's body. This hypothesis is confirmed by the subcutaneous dark red haematomas of the pelvic limbs, which were apparently formed as a result of the animal's body hitting hard surfaces during agonal convulsions at the time of the terminal pause.

Considering the above, we propose to detail the classification of animal deaths from mechanical asphyxiation depending on the nature of the traumatic factor and the place of its impact, with consideration of the case file. Considering the thanatological cases from our expert practice (Table 1), asphyxiological death of an animal can occur in cases of neck strangulation (e.g., with a rope), chest compression (e.g., with hands), obstruction of the respiratory tract (e.g., with dense objects), or aspiration (e.g., with liquids).

The thanatogenesis of mechanical asphyxia can be logically represented in two parts: firstly, the cessation of free air access causes the development of acute anoxia of the medulla oblongata, and secondly, damage to the neck stimulates vagal reflexes, causing sudden myocardial paralysis due to the development of bradycardia. However, in the premortem state, according to our clinical observations, the absence of respiratory excursions of the animal's chest precedes cardiac paralysis, which should

be considered from the standpoint of view of the semiotics of rapid death.

Therefore, when arguing for a forensic veterinary diagnosis of asphyxiated animal death, it is advisable to consider the genesis of death, where the initial (determining) and immediate causes of death coincide, while fatal complications in the form of cerebral anoxia and bradycardia are, in the causal aspect, competing links in thanatogenesis. Undoubtedly, in case of rapid death of animals, there is a need, firstly, to identify criteria that impartially substantiate the conclusion of a forensic veterinary expert, and secondly, to differentiate mechanical asphyxiation from other comparable conditions that result in sudden death.

It is during the implementation of the mechanism of animal death caused by asphyxiation that general signs are formed, which are proposed to be recorded at the relevant stages

of forensic veterinary examination of the animal cadaver.

Thus, to substantiate the evidence of an animal's death from a specific type of asphyxia, it is logical to describe, first of all, the external general asphyxial signs (patterns of the first level of evidence). Thus, during the examination of the experimental cadaver of a dog whose death occurred as a result of *neck strangulation* by the locking system of a device for remote fixation of dogs, attention is drawn to the pronounced swelling of the muzzle, hyperaemia of the conjunctiva and sclera in the form of subconjunctival petechiae (Fig. 35), rounded small-point haemorrhages with a diameter of 0.3 cm in the area of the tongue root and damage from the teeth to the back of the tongue (Fig. 36), prolapse of the rectum due to relaxation of the sphincters (Fig. 37). W.-H. Huang *et al.* (2021) described death in such situations.

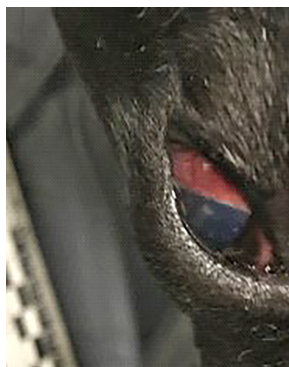


Figure 35. Conjunctival petechiae of the right eye of a dog cadaver in case of death from strangulation asphyxia

Notes: macro photo

Source: developed by the author of this study



Figure 36. "Pinching" of the tongue and haemorrhage of the oral mucosa of a dog cadaver in case of death from strangulation asphyxia

Notes: macro photo

Source: developed by the author of this study

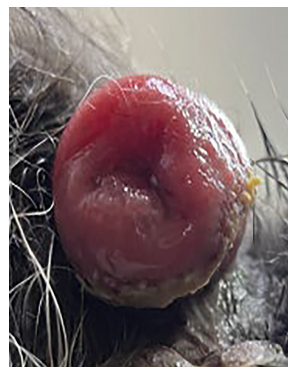


Figure 37. Prolapse of the rectum of a dog cadaver in case of death from strangulation asphyxia

Notes: macro photo

Source: developed by the author of this study

When determining the cause of death of a dog with external signs of violent death, the position of the cadaver's head with its unnaturally increased mobility against the background of

an unexpressed postmortem contracture of the neck muscles is of particular note. External bleeding that contaminates the scalp is also caused by trauma to the tongue by canines (Fig. 38).

Radiological examination of the neck of the experimental dog cadaver in RL projection indicates anatomical instability of the cervical vertebrae at the level of the *articulatio atlantooccipitalis* (Fig. 39). Histotopograms of skeletal muscles obtained from the area of neck

strangulation confirm signs of local soft tissue compression: thinning, flattening, and pressing of muscle fibres against each other, their dystrophy, compaction, compression, and desolation of blood vessels, waviness and rupture of myofibrils (Fig. 40.1).

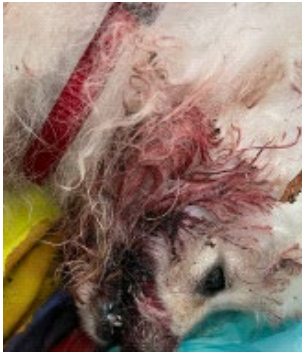


Figure 38. Contamination of the dog's head with blood due to strangulation asphyxia

Notes: macro photo

Source: developed by the author of this study



Figure 39. Rupture of the atlanto-occipital joint of a dog cadaver

Notes: photographic screen of X-ray in RL projection (X-ray Device PCMAX-40HP)

Source: developed by the author of this study

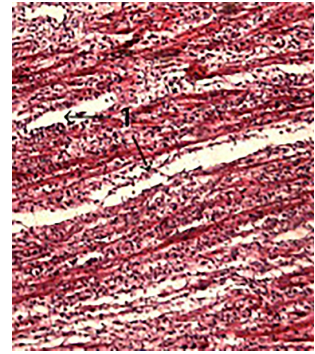


Figure 40. Histotopogram of the muscle tissue of the strangulated area of the neck of a dog cadaver due to local compression

Notes: 1 – deformation and tearing of muscle fibres. Haematoxylin and eosin, 10×40, (microscope "Granum R50")

Source: developed by the author of this study

As for the peripheral organs of haemopoiesis, diametrically opposite morphological changes were observed in them. Thus, histological examination of the mandibular lymph nodes located topographically above the locus of strangulation revealed haemorrhages in the tissue above their capsule with simultaneous severe hyperaemia of the venules around and directly in the capsule of these nodes. The vessels of the lymph node are dilated and filled with blood cells, the focuses of microdiapenic haemorrhages are distributed evenly over the entire area of the histological specimen (Fig. 41.1), and the lumen of the vessel is filled with conglomerates of erythrocytes (Fig. 41.2).

The histomorphological examination of the spleen revealed the absence of pronounced microscopic changes. The outside is covered with a capsule made of dense fibrous fabric, which can be easily removed. Trabeculae, containing blood vessels, smooth muscle fibres and nerves, extend from the capsule. The white pulp covers a large area. It is organised into cylindrical clutches of lymphocytes that surround the central arteries that form the periarterial lymphoid sheaths. The red pulp consists of sinusoidal vessels and pulp bundles located between them. However, the segmental section shows visual anaemia (Sabinsky sign) and a slight scraping, which is confirmed by the findings

of histomorphological studies (Fig. 42). The erythrocytes are located in small clusters over the histotopogram area, unevenly (Fig. 43.1). During the forensic veterinary examination

of the cadavers of animals whose deaths were caused by mechanical asphyxiation, general signs of asphyxiation of varying degrees of severity were identified.

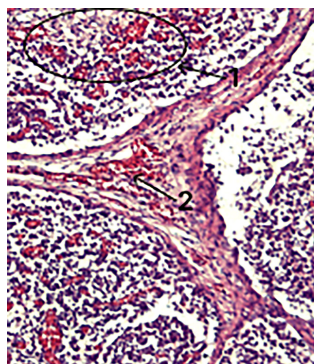


Figure 41. Histotopogram of a lymph node of a dog cadaver in case of death caused by strangulation asphyxia

Notes: 1 – diapedetic haemorrhages; 2 – erythrocyte conglomerates in the vessel lumen. Haematoxylin and eosin, 10×40, (Granum R50 microscope)

Source: developed by the author of this study



Figure 42. Segmental section of the spleen of a dog cadaver in case of death caused by strangulation asphyxia

Notes: macro photo

Source: developed by the author of this study

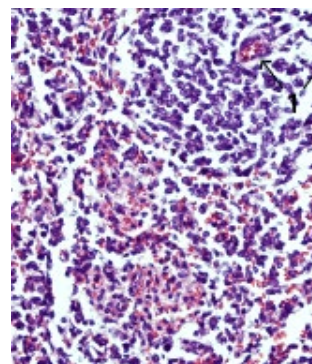


Figure 43. Histotopogram of the spleen of a dog cadaver in case of death caused by strangulation asphyxia

Notes: 1 – uneven accumulation of red blood cells. Haematoxylin and eosin, 10×40, (Granum R50 microscope)

Source: developed by the author of this study

In such cases, it is crucial to adequately interpret and distinguish between them, which is particularly true for the statement of internal general asphyxiological features (patterns of the first level of evidence). Specifically, the absence of dark red blood clots in large vessels, an “asphyxiated heart” due to blood stasis in its right half, venous hyperaemia of internal organs, multifocal spotted or punctate haemorrhages (Tardieu spots) under the epicardium (Fig. 44) and lung pleura (Fig. 45), emphysema with numerous ruptures of alveolar membranes and haemorrhages in them (Fig. 46.1) on a small number of intact structural elements of the parenchyma (Fig. 46.2).

In the array of animal deaths from mechanical asphyxiation, fatal neck strangulation

ranks second. Notably, imitation of killing an animal by hanging has been encountered in our forensic practice. In contrast to humans, among whom hanging is one of the most common causes of violent death (Marrone *et al.* 2023), strangulation asphyxiation in animals is predominantly the result of cruelty. During the experimental suspension of animal cadavers two hours after death, local signs of strangulation were observed, specifically, subcutaneous haematomas, mucosal oedema, and skin compression, which is caused by the prolonged vertical position of the cadaver in the noose under the influence of gravity. Unlike the human neck, the neck of animals is densely covered with hair, which clearly determines the absence of a pronounced classical strangulation furrow, which

is not a permanent feature, but, accordingly, the main sign of death from strangulation. In this regard, the forensic veterinary expert's special attention should be focused on establishing the signs of intravital formation of fatal injuries. Thus, during the dissection of the neck skin of

a dog whose death was reliably caused by strangulation asphyxia, there was a spilled blood imbibition of the muscles and subcutaneous tissue with a characteristic relief impression from the laryngeal cartilage and dermal oedema below the strangulation locus (Fig. 47).

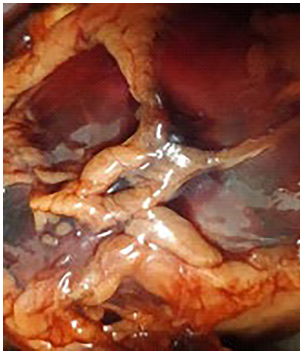


Figure 44. Spotted haemorrhage under the epicardium of a dog cadaver in case of death from asphyxiation

Notes: macro photo

Source: developed by the author of this study

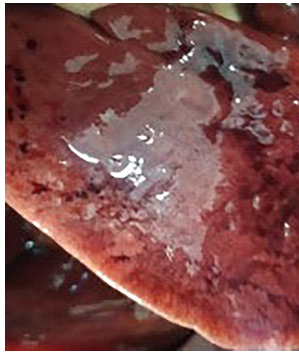


Figure 45. Point haemorrhages under the lung pleura of a dog cadaver in case of death from asphyxiation

Notes: macro photo

Source: developed by the author of this study

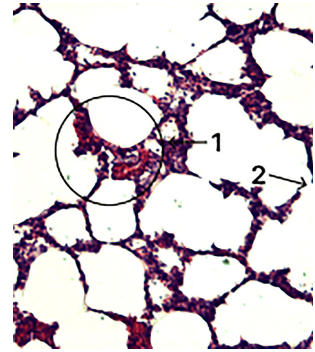


Figure 46. Histotopogram of the lungs of a dog cadaver in case of death from asphyxia with alveolar emphysema

Notes: 1 – haemorrhage and destruction of the alveolar membranes; 2 – intact alveolus. Haematoxylin and eosin, 10×40, (Granum R50 microscope)

Source: developed by the author of this study



Figure 47. Haematoma of the locus of strangulation of a dog cadaver

Notes: macro photo

Source: developed by the author of this study

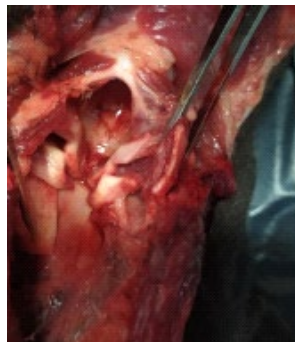


Figure 48. Blood leakage into the larynx of a dog cadaver due to strangulation asphyxia (sagittal section)

Notes: macro photo

Source: developed by the author of this study

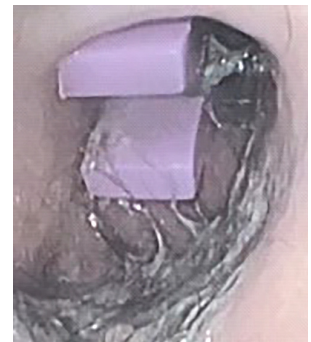


Figure 49. Foreign body in the laryngeal cavity of a dog cadaver

Notes: photo screen of endoscopic examination (Olympus CLV-160)

Source: developed by the author of this study

A sectional examination of the larynx of an experimental dog cadaver revealed signs of blood leakage into the cavity itself (Fig. 48), caused by damage to the surrounding blood vessels. In the absence of reliable macroscopic signs of intravital strangulation, we emphasise that the morphological picture of an animal cadaver must be supported by the results of histomorphological studies that reasonably document pathological patterns at the micro level, and a forensic veterinary expert can only reach a reasoned conclusion by considering structural changes at different levels of organisation, only in totality.

Summarising the above, we emphasise that in the death of animals caused by strangulation asphyxia, apart from general asphyxial signs, subspecific signs (patterns of the second level of evidence) were identified, namely, “pinching” of the tongue between the teeth, blood leakage from the mouth and nose above and below the locus of injury, hyperaemia of the nearest lymph nodes, spleen anaemia, diapedetic haemorrhages in the mucous membrane of the tongue root, deformation of the skeletal muscle fibres of the animal’s neck at the locus of injury, which should be considered in the practice of forensic veterinary examination. N.M. Mazzante *et al.* (2020) cited cases of lethal strangulation of the neck of animal cadavers with intimal tears, tears of the carotid arteries and jugular veins, damage to the ligaments and muscles of the neck, cartilage of the larynx and trachea, and the hyoid apparatus. In our expert practice, we have not detected the specified phenomena.

Structural and functional changes that occur in rare accidents of mechanical obstruction of air access caused by obstruction of the respiratory tract of animals by foreign objects are conditioned by the direct effect of the obstructing factor on the laryngeal interoceptors, followed by the development of a reflex response in the form of a combination of acute haemodynamic disorders and metabolic syndrome, which together lead to rapid death (Fig. 49).

During the forensic veterinary internal examination of the cadavers of such animals, only general asphyxiological signs are found.

From the standpoint of forensic veterinary examination, it stays relevant to establish subspecies-specific signs of death caused by *aspiration asphyxiation*, since drowning, as a separate variant of animal killing, is most common in expert practice. In this context, the death of an animal as a result of fluid aspiration can occur in certain pathological conditions or processes; however, such a lethal outcome is classified as a non-violent death and occurs mainly during hospitalisation in veterinary medicine. However, in case of suspicion of violent death caused by drowning, the forensic veterinarian should pay special attention to the argumentation of a direct causal link, since, according to our observations, during a long stay of the animal cadaver in water, the fluid can fill the lung parenchyma through direct entry into the respiratory tract or by diffusion caused by emphysematous biotransformation.

Thus, during the external examination of the examinee cadaver of a kitten whose death occurred due to drowning and which was removed from the water immediately after death, a fine bubbly light-coloured foam around the nostrils and mouth was visually noticeable (Fig. 50).

The autopsy of the respiratory tract shows that similar fluid also fills the lumen of the trachea, but in the bifurcation area and below, in the bronchi, it has a pinkish tint (Krushevsky sign). The lungs appear to be enlarged in volume, with pronounced rounded edges, “marbled” in colour (Fig. 51): light red areas alternate with light grey oval spots. The latter are subpleural haemorrhages (Rasskazov-Lukomsky-Paltauf sign) and are formed as a result of destruction of pulmonary acini capillaries due to excessive stretching and rupture in the setting of acute alveolar emphysema. A pink, foamy liquid may flow freely from the surface of the lung incision.

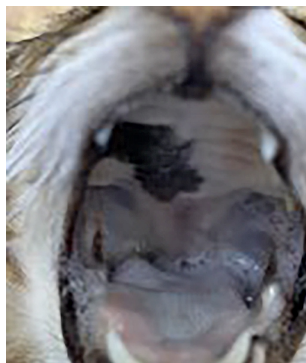


Figure 50. Light foamy liquid in the mouth of a kitten cadaver in case of death by drowning

Notes: macro photo

Source: developed by the author of this study

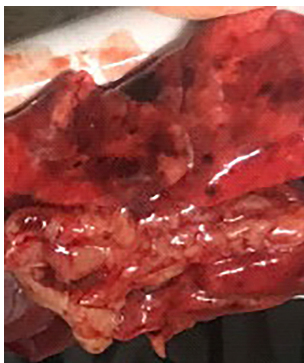


Figure 51. Pronounced haemorrhages under the lung pleura of a kitten cadaver in case of death by drowning

Notes: macro photo

Source: developed by the author of this study

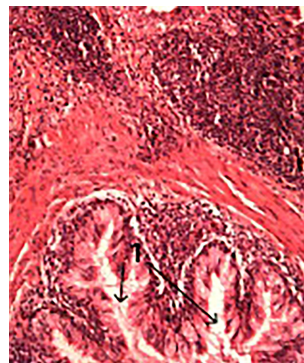


Figure 52. Histotopogram of pulmonary acinus of a kitten cadaver in case of death by drowning

Notes: 1 – the lumen of the bronchioles is filled with light fluid. Haematoxylin and eosin, 10×40, (Granum R50 microscope)

Source: developed by the author of this study

Histomorphologically, the lumen of the bronchioles was found to be filled with fluid with red blood cells (Fig. 52.1), and the alveolar vesicles were in a state of congestive hyperaemia, which was confirmed by G. Piegari *et al.* (2019), who obtained analogous macroscopic and microscopic findings.

Thus, the signs found in the experimental cadaver prove the death of the animal by thanatogenesis of aspiration asphyxia caused by drowning. In terms of the death of animals caused by asphyxiation as a result of *compression of the chest cavity*, in the practice of forensic veterinary examination, such cases are rare. This was confirmed by Kr. Prihirunkit (2022) in an alternative study of dog cadavers that died due to chest compression by hands. Undoubtedly, excessive compression of the animal's chest limits the number of respiratory excursions and, subsequently, due to pressure, disrupts lung vascularisation. Pressure on the chest contributes to an increase in partial pressure in the pleural cavities, is accompanied by overstretching and rupture

of the microcirculatory vessels and causes the development of acute hypoxia in the setting of hypercapnia.

The forensic veterinary diagnosis of compression asphyxia can be made only in the absence of “active” organ nosology, prolonged crush syndrome, gross trauma to the chest, or penetrating injuries to the chest, as such injuries cause pulmonary atelectasis, which has independent thanatogenetic significance. Thus, during the external examination of the experimental cadaver of a neonatal puppy that died due to an accident caused by another dog squeezing the abdomen and thorax in the anteroposterior direction, the presence of subconjunctival haemorrhages and an “ecchymotic mask” of the skin of the oronasal part of the head and distal thoracic limbs was detected (Fig. 53).

Cardiosternal contact, verified by radiological examination of the chest of the cadaveric puppy in the RL projection, indicates no signs of pneumothorax (Fig. 54). The autopsy data confirm the expert hypothesis of compression of the chest cavity organs, as the cadaver

revealed massive dark red infarcts in the cranial lobes of the lungs, relief of the inner surfaces of the lungs from the heart, their atelectasis

against the background of venous hyperaemia, and a liquid state of blood in the heart chambers and large blood vessels (Fig. 55).



Figure 53. “Ecchymotic mask” of the head and thoracic limbs of a puppy cadaver in case of death from compression asphyxia

Notes: macro photo

Source: developed by the author of this study

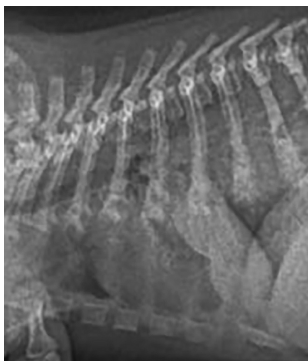


Figure 54. Pulmonary atelectasis of a puppy cadaver in case of death from compression asphyxia

Notes: photographic screen of radiograph in RL projection (X-ray Device PCMAX-40HP)

Source: developed by the author of this study



Figure 55. Appearance of the organ complex of the chest cavity of a puppy cadaver in case of death from compression asphyxia

Notes: macro photo

Source: developed by the author of this study

Thus, an objective basis for establishing causal relationships in critical conditions of animals with fatal consequences of mechanical and thermal trauma and asphyxiation is the establishment of morphological positions of fatal injury, reflected in the form of a forensic veterinary diagnosis, expressed in thanatogenetic and nosological forms, integrated with each other. From the standpoint of evidence-based veterinary medicine, we consider it necessary to selectively distinguish all morphological signs identified during the forensic veterinary examination of an animal cadaver by levels with the obligatory substantiation of the thanatognomic criteria inherent in a particular subspecies of violent animal death.

Thus, when a forensic veterinary expert is arguing for a conclusion on the cause of death, we believe that it is necessary to rely on the main links of thanatogenesis to resolve certain

issues in the construction of the diagnosis, considering the semiotics of the injury. Considering the above, it is advisable to distinguish the initial cause of death of an animal according to the principle of complete completion of the links of the intra-nosological cause-effect relationship: the main cause of death is considered to be a nosological unit that, through the cause-effect relationship of pathomorphogenesis, ends with the direct cause of death.

Conclusions

For the first time in Ukraine, the authors of this study substantiated the cause-and-effect relationships in the genesis of violent death of animals caused by haemodynamic disorders and hypoxemia based on the findings of original research using the proposed approach with the identification of thanatognomic patterns according to a two-level system of

evidence. It was substantiated that irreversible disorders of haemodynamics and hypoxia are involved in the thanatogenesis of animal death caused by mechanical injuries, thermal trauma, and mechanical asphyxiation. Based on such approach, it is necessary to assess the area of pathological changes in internal organs that are incompatible with life and to build an “expert hypothesis” about the determining cause of the animal’s death.

To follow the principles of evidence-based veterinary medicine, we consider it necessary to selectively distinguish all morphological positions identified during the forensic veterinary examination of an animal cadaver into two levels with the obligatory substantiation of the thanatognomonic criteria inherent in a particular subtype of violent death of an animal.

The patterns of rapid death of animals of the first level of evidence common to the fatal consequences of mechanical trauma and mechanical asphyxiation were established as follows: absence of blood clots in large-calibre vessels, congestive venous hyperaemia of internal organs, multifocal spotted or punctate haemorrhages (Tardieu spots) under the epicardium and pleura of the lungs, alveolar emphysema of the lungs.

Proven patterns of mechanical asphyxia of the second level of evidence: “pinching” of the tongue between the teeth, blood leakage from the mouth and nose above and below the locus of injury, hyperaemia of the nearest lymph nodes, Sabinsky sign, diapedetic haemorrhages in the mucous membrane of the tongue root, deformation of the skeletal muscle fibres of the animal’s neck at the locus of injury, Krushevsky sign, Rasskazov-Lukomsky-Paltauf spots.

The cause of death of the animals due to generalised overheating as a result of brain swelling and hyperaemia of its membranes was substantiated. A reliable sign of death in the case of general hypothermia, the Wischnewsky spots, was found.

Specific signs of traumatic injury (patterns of the second level of evidence) were identified: pleomorphism of the body injury, acute blood loss, general anaemia of the circulatory anaemic type. It was found that the most common adverse effects of traumatic illness in animals are caused by life-threatening injuries, namely: gross anatomical destruction (crushing or separation) of the body, significant damage to vital organs (e.g., heart tamponade with blood due to extra-mural infarction, brain and/or heart contusion); acute bleeding: external and internal; obstruction of major vessels by traumatic emboli (air, tissue, etc.); mechanical asphyxia: aspiration of the respiratory tract with blood and traumatic laryngeal edema; combined polytrauma; incompatible functional disorders caused by acute organ failure and/or pain shock. The new data obtained should be implemented in the practice of forensic veterinary examination of animal cadavers.

The prospect of further research will be the application of the thanatogenetic approach to the formulation of a forensic veterinary diagnosis in cases of fatal haemostasis and cardiac death of animals.

Acknowledgements

None.

Conflict of Interest

None.

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Танатогномонічне обґрунтування судово-ветеринарного діагнозу за смерті собак і котів внаслідок розладів гемодинаміки та гіпоксемії

Роман Казанцев

Аспірант

Державний біотехнологічний університет
62341, вул. Академічна, 1, с. Мала Данилівка, Україна
<https://orcid.org/0000-0002-4479-1516>

Іван Яценко

Доктор ветеринарних наук, професор
Державний біотехнологічний університет
62341, вул. Академічна, 1, с. Мала Данилівка, Україна
<https://orcid.org/0000-0001-8903-2129>

Анотація. Нині в Україні відсутні юридично закріплені регламентовані процедури проведення судово-ветеринарної експертизи трупів тварин, що ускладнює об'єктивну кваліфікацію правоохоронними органами форм адміністративних та кримінальних правопорушень у рамках досудового розслідування. А тому, особливої актуальності набувають науково обґрунтовані результати дослідження у сфері поводження з тваринами. У роботі поставлено за мету – обґрунтувати причинно-наслідкові зв'язки у генезі насильницької смерті тварин внаслідок механічних ушкоджень, термічної травми та механічної асфіксії з виокремленням танатогномонічних патернів за дворівневою системою доказовості. Використовували методи комплексного судово-ветеринарного дослідження трупів тварин із наступною логіко-гносеологічною обробкою результатів. Аргументовано, що головними ланками танатогенезу у разі смерті тварин від зазначених видів є незворотні порушення гемодинаміки та гострої гіпоксемії. На підставі такого підходу, запропонована оцінка площі несумісних із життям патологічних ушкоджень внутрішніх органів із подальшою «експертною гіпотезою» щодо визначальної причини смерті тварини. Виокремлені морфологічні позиції судово-ветеринарного діагнозу у вигляді танатогномонічних позицій, притаманних певному підвиду насильницької смерті тварини, за двома рівнями доказовості та доведена їх критеріальна інформативність. Встановлені патерни швидкої смерті тварин першого рівня доказовості, спільні для фатальних наслідків механічної та термічної травми і механічної асфіксії: «асфіктичне серце», застійна венозна гіперемія внутрішніх органів, ознака Тард'є, альвеолярна емфізема легенів. Селективно рандомізовані патерни другого рівня доказовості,

показана їх видоспецифічність. Деталізовані обставини, за яких у тварин виникають фатальні механічні і термічні ушкодження та асфіксія, обґрунтовані механізми настання смерті. Акцентована увага судово-ветеринарних експертів на особливостях аргументації у висновку необхідного прямого причинно-наслідкового зв'язку генезу насильницької смерті. Отримані результати з дотриманням стандарту доказовості «дослідження об'єкта судово-ветеринарної експертизи на різних морфологічних рівнях» рекомендуються до впровадження у практику судово-ветеринарної експертизи трупів тварин

Ключові слова: тілесні ушкодження; термічна травма; механічна асфіксія; танатогенез тварин; аргументація висновку експерта



2024. Volume 15, No. 2. P. 102-120

Received: 08.02.2024 Revised: 19.04.2024 Accepted: 29.05.2024

DOI: 10.31548/veterinary2.2024.102

UDC 636.09:599.323.41:591.473.3:609.2

Influence of allogeneic mesenchymal stem cells of red bone marrow culture on the development of Lewis lung epidermoid carcinoma *in vivo*

Larysa Kladnytska*

Doctor of Veterinary Sciences, Professor
National University of Life and Environmental Sciences of Ukraine
03041, 15 Heroiv Oborony Str., Kyiv, Ukraine
<https://orcid.org/0000-0002-9360-0587>

Viktor Tomchuk

Doctor of Veterinary Sciences, Professor
National University of Life and Environmental Sciences of Ukraine
03041, 15 Heroiv Oborony Str., Kyiv, Ukraine
<https://orcid.org/0000-0003-0622-6345>

Vladyslav Velychko

Postgraduate Student
National University of Life and Environmental Sciences of Ukraine
03041, 15 Heroiv Oborony Str., Kyiv, Ukraine
<https://orcid.org/0000-0002-5227-9168>

Volodymyr Salata

Doctor of Veterinary Sciences, Professor
Stepan Gzhytskyi National University of Veterinary Medicine and Biotechnologies
79010, 50 Pekarska Str., Lviv, Ukraine
<https://orcid.org/0000-0002-7175-493X>

Jakov Šengaut

Doctor of Philosophy in Veterinary Sciences
Lithuanian Small Animal Veterinary Association
LT-03147, 1 Gerosios Vilties Str., Vilnius, Lithuania
<https://orcid.org/0000-0002-5471-0046>

Suggested Citation:

Kladnytska, L., Tomchuk, V., Velychko, V., Salata, V., & Šengaut, J. (2024). Influence of allogeneic mesenchymal stem cells of red bone marrow culture on the development of Lewis lung epidermoid carcinoma *in vivo*. *Ukrainian Journal of Veterinary Sciences*, 15(2), 102-120. doi: 10.31548/veterinary2.2024.102.

*Corresponding author



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Abstract. The relevance of this study is conditioned by the widespread use of stem cells in veterinary medicine, a wide range of studies and ambiguous data on the oncoprotective properties of stem cells of different origins. In this regard, the purpose of this study was to investigate the course of the tumour process in Lewis lung carcinoma and the specific features of the effect of allogeneic mesenchymal stem cells of red bone marrow culture on it. The leading approach to investigating this problem was the method of modelling Lewis lung carcinoma in C57BL6 mice and the use of stem cells. The use of allogeneic mesenchymal stem cells from the bone marrow culture of C57BL6 mice with transplanted epidermoid metastatic carcinoma of the Lewis lung contributed to the activation of the tumour process. Under the influence of allogeneic mesenchymal stem cells of red bone marrow culture from Day 14 to Day 24 of the study, the body weight of mice decreased by 7.0-12.1% ($P < 0.05$) compared to the control, the diameter of the primary tumour increased by 1.43-1.51 times ($P < 0.05$), which is conditioned by the activation of primary tumour growth. The number of lymphocytes as producers of vascular growth factor in primary tumour tissue under the influence of allogeneic mesenchymal stem cells of red bone marrow culture significantly increased by 1.47 and 1.52 times on Day 18 of the experiment compared to animals of the control group and placebo ($P < 0.05$), respectively. This promoted angiogenesis in the primary tumour node and metastasis through the circulatory system. After administration allogeneic mesenchymal cells of red bone marrow culture to mice, a larger volume of lung metastases was recorded, which was $41.52 \pm 7.9 \text{ mm}^3$ compared to the values in the control and placebo groups, respectively, 17.94 ± 6.59 and $16.43 \pm 5.32 \text{ mm}^3$. The morphological picture of the histological sections of the primary tumour of Lewis lung carcinoma confirms all the signs of qualitative and quantitative indicators of its progression. The findings obtained are of both theoretical and practical value for clinical veterinary medicine on the use of allogeneic bone marrow mesenchymal stem cells in tumour processes

Keywords: primary tumour; infiltration; lymphocytes; metastases; vascularisation; C57BL6 mice

Introduction

Mesenchymal stem cells (MSCs) are multipotent stem cells with significant potential for differentiation and self-renewal. The therapeutic properties of MSCs are associated with the potential for transdifferentiation, secretion of trophic factors, and immunomodulation. MSCs differentiate into numerous mesenchymal cell lineages, including adipocytes, osteocytes, chondrocytes, pericytes, and fibroblasts. According to H. Yang *et al.* (2022), they are also able to transform into epithelial (endodermal) and neural (ectodermal) lineages, into hepatocytes under certain conditions of in vitro culture, and are involved in maintaining tissue structure and controlling inflammation.

Overall, MSCs have a beneficial effect through immunomodulatory regulation and paracrine mechanisms. Y. Xia *et al.* (2023) report that MSCs have been shown to have a therapeutic effect in many diseases, such as myocardial infarction, liver cirrhosis, limb ischaemia, nervous injury, among others. In general, MSCs maintain tissue integrity as the key regulators of tissue homeostasis. Apart from their normal distribution in individual organs, they accumulate in damaged tissues, promoting tissue regeneration. In response to tissue damage, MSCs are mobilised from their niches by relevant signals and migrate to take part in tissue regeneration and remodelling. Certain

cultivation factors and body functioning conditions can increase their intake into the bloodstream and damaged tissues. It is also known that MSCs can be localised together and form the core of blood clots, which leads to circulatory disorders. In addition, L. Frisbie *et al.* (2022) note that MSCs are tumourigenic due to their reproductive properties and the ability to provoke acute or chronic immunogenicity of cells as foreign agents in the body.

The development and progression of a tumour is determined not only by the tumour cells involved, but also by the tumour microenvironment. The cell populations surrounding tumours can interact indirectly or directly with tumour cells and significantly affect their functions and properties. In particular, MSCs play an important role in tumour development, demonstrating various variants of intercellular interaction through cytokines, chemokines, growth factors, which can ultimately trigger the formation of a tumour stem cell pool (TSCP) or the reproduction of new tumour cell populations.

X. Xuan *et al.* (2021) reported that MSCs had multifaceted effects on tumour cells, mainly promoting tumour growth through their role in regulating inflammation and tissue repair. According to G. Zhang *et al.* (2020), mesenchymal stem cells support tumour growth through various mechanisms, including promotion of drug resistance, activation of metastasis, pro-angiogenic effects, enrichment of the cancer stem cell niche, and high immuno-inhibitory properties.

According to G. Guo *et al.* (2021), MSCs also affect the survival and stemness of tumour cells and promote the growth of the tumour vasculature, produce angiogenic factors, and differentiate into pericytes. Mesenchymal stem cells promote tumour cell motility and secrete cytokines (IL-6), chemokines (CXCL1, CXCL2, and CXCL12), and several matrix metalloproteinases that facilitate tumour cell migration. According to C.R. Harrell *et al.* (2021), they have an

important immunomodulatory function, which is primarily immunosuppressive. M. Timaner *et al.* (2020) suggest that MSCs can enhance the resistance of tumour cells to anticancer drugs, at least in part, by releasing exosomes containing numerous mediators, including microRNAs, which can alter the properties of tumour cells. Thus, in most studies, MSCs have a pro-tumour effect, but there are studies that have shown that they can also exert an anti-tumour effect by inhibiting Wnt signalling, suppressing angiogenesis, stimulating inflammatory cell infiltration, inducing cell cycle arrest and cause apoptosis. Z. Chen *et al.* (2021) proved that IFN- β is synthesised by adipose tissue-derived MSCs cultured at high cell density, which reduces the growth of breast cancer cells – MCF-7.

Y. Stepanov *et al.* (2021) found that the route of administration of mesenchymal stem cells – intramuscular or intravenous – also has an impact on tumour growth and angiogenesis. Specifically, intramuscular transplantation of placenta-derived stem cells slows down primary tumour growth and angiogenesis compared to the control on Day 15 of the study, while intravenous injection of stem cells stimulates these processes. On Day 23 of the tumour process, no significant changes in tumour growth were observed in animals of both groups, in contrast to angiogenesis, which was slowed or activated, respectively. The number and volume of metastases in both groups of animals was higher than in the control. These findings suggest a bidirectional effect on the development of the tumour process in the early period of development. However, regardless of the route of MSC transplantation, tumour progression, and activation of metastasis are evident.

To obtain enough material for transplantation, scientists have developed methods to activate the proliferation of stem cells during *in vitro* cultivation. Thus, A. Mazurkevych *et al.* (2021) propose to activate proliferation using

different concentrations of insulin-like growth factor (IGF-1), growth hormone (rhGH), fibroblast growth factor (FGF-2), and biolaminin 521 LN. The findings obtained upon the use of stem cell growth activators were ambiguous, namely, IGF-1 had a positive effect on proliferative activity, while the effect of rhGH decreased the proliferative index. However, both growth factors did not affect genetic changes in cells.

Thus, considering the numerous and ambiguous results found in the study of the effect of MSCs on the tumour process, the study of carcinogenesis, metastasis, changes in the skeletal muscle of mice with transplanted Lewis lung carcinoma under the influence of allogeneic mesenchymal stem cells of bone marrow culture is a relevant issue.

The aim of the study is to determine the effect of allogeneic mesenchymal stem cells on the oncological process in the experimental reproduction of Lewis lung carcinoma.

Literature Review

The principal mechanisms of MSCs influence on the oncological process include stimulation of angiogenesis; changes in the biological properties of tumour cells, specifically sensitivity to apoptosis induction; effect on immunocompetent cells, modulation of epithelial-mesenchymal transition. X. Li *et al.* (2022) noted that carcinoma growth can be considered the result of a “war” or “alliance” due to the intercellular signalling observed between different cell types. Cancer cells secrete chemokines and cytokines, such as CCL2 and TGF- β , which are involved in the activation and retention of mononuclear inflammatory cells and tumour-associated fibroblasts, as well as in the carcinogenic transformation of macrophages. Furthermore, as noted by N. Wu *et al.* (2020), a complex and dynamic interaction occurs after stromal cell retention. MSCs play a key role as they interact with

stromal cells and cancer. The multidirectional signals between these cells are mediated by soluble factors, integrins (apoptotic bodies, microvesicles and exosomes), which leads to a special nanocommunication between different types of tumour cells. G. Jothimani *et al.* (2022) proved that exosomes, the smallest subgroup of EVs (30-150 nm in diameter), are surrounded by a protein-phospholipid bilayer membrane, and its lumen partially repeats the contents of the parent cell (DNA, microRNA, matrix RNA (mRNA), growth factors, nucleic acids, chemokines, and cytokines).

G. Jothimani *et al.* (2022) found that under physiological conditions, MSCs produce many exosomes (MSCs-Ex). MSC-Ex include a powerful set of molecules, including more than 150 different microRNAs and more than 850 unique gene products. Thus, MSC-Ex can induce various cellular responses in a wide range of cells that are responsible for modulating many physiological functions. In the study by S. Guo *et al.* (2021) found that MSCs are recipients of impulses from the tumour, but thanks to MSC-Ex, MSCs have the ability to interact with several cell types in the tumour microenvironment, thereby inducing phenotypic and functional changes that can have a significant impact on tumour growth. Similar to MSCs, some types of MSCs-Ex exhibit the ability to support and induce tumour growth, metastasis and invasion, while other MSCs-Ex have antitumour effects, which are related to their origin and tumour type.

Lewis lung carcinoma is a spontaneous tumour isolated from C57BL/6 Margaret Lewis mice and maintained by subcutaneous or intramuscular transplantation since 1951 and is widely used in experimental pathology. In the subcutaneous transplantation of Lewis lung carcinoma culture cells, both the primary tumour and its metastases showed a comparable histological aspect. The primary tumour (primary tumour node) is formed at the transplant

site, penetrating the border subcutaneous tissue and muscles. The primary tumour contains large cells without clear differentiation – undifferentiated carcinoma. The cells are large, rounded or irregularly shaped with an eosinophilic cytoplasm and usually a large, rounded nucleus. Atypical mitoses were recorded in each field of view of the slide. In the primary tumour and its metastases, giant cells with one or more hyperchromatic nuclei were detected. Extensive necrosis of the central area of the tumour was recorded. The presence of a thin, unevenly distributed fibrillar stroma can be observed by staining with Gomori reticulin.

H. Kuroda *et al.* (2021) proved that mesenchymal stem cells are localised in breast carcinoma, where they are incorporated into the tumour-associated stroma. However, the involvement of MSCs, including their derivatives, in the pathophysiology of carcinoma has not been considered. The authors found that human bone marrow-derived MSCs, when added to mildly metastatic mammary tumour cells, cause a significant increase in the metastatic activity of cancer cells. If this cellular mixture is injected into the subcutaneous area, the formation of a xenograft tumour is recorded. Breast carcinoma cells increase the *de novo* supply of the chemokine CCL5 from MSCs, which subsequently has a paracrine effect on cancer cells, stimulating their invasion, motility and metastasis. This enhanced metastatic capacity is reversible and depends on CCL5 signalling through the chemokine receptor CCR5. Taken together, these data provide evidence that the tumour microenvironment promotes the spread of metastases, causing reversible changes in the phenotype of cancer cells.

L. Sun *et al.* (2020) noted that current therapeutic strategies for cancer treatment are usually insufficient to completely destroy malignant cells, as cancer stroma cells are therapeutically resistant. E. Sahai *et al.* (2020) found that

cancer-associated fibroblasts (CAFs) primarily represent a heterogeneous type of stromal cell and are important components of the tumour microenvironment (TME). CAFs are the most common type of stromal cells that contribute to tumour progression through specific intracellular mechanisms and their ability to influence other cell types. Recent studies have highlighted a new function of CAF in the remodelling of TMEs, which contributes to tumour progression and affects treatment response through various molecular mechanisms. The complex mechanism of CAF activity, which promotes tumour development, can be used to improve the efficiency of diagnostic and therapeutic procedures.

Materials and Methods

The experiments were conducted on the basis of the scientific laboratory of the Department of Animal Biochemistry and Physiology named after Academician M.F. Gulyi of the NUBiP of Ukraine during 2019-2023 on male mice of the C57Bl/6 line. The mice were 2.0-2.5 months old and weighed 19.0-22.0 g. Mice were kept under standard vivarium conditions with natural light on a full diet, with water freely available. The study followed the ARRIVE guidelines and was conducted according to the guidelines of the EU Directive 2010/63/EU on the protection of animals used for scientific purposes (Directive 2010/63/EU..., 2010; Percie du Sert *et al.*, 2020).

For the experimental studies, the tumour process of Lewis lung carcinoma (LCC), which metastasises to the lungs, was modelled. For this, Lewis lung epidermoid carcinoma cells were used, which were obtained from the cell bank of human and animal tissue lines of the R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology of the National Academy of Sciences of Ukraine (Kyiv).

Before transplantation, Lewis lung carcinoma cells were cultured under standard conditions in Dulbecco's Modified Eagle Medium

(DMEM) with 3-5% foetal bovine serum (FBS) and 1% antibiotic antimycotic (Sigma, USA). Cultivation was carried out in a CO₂ incubator at absolute humidity, 37°C and 5% CO₂. After forming a monolayer of cells, the biomaterial was seeded and cultured under analogous conditions to obtain a sufficient number of cells for transplantation. Cells were suspended using detergents under the control of an inverted Carl Zeiss 40 microscope (Germany).

Obtaining allogeneic mesenchymal cells of red bone marrow culture (BM-AMSCs). These cells were obtained by washing out red bone marrow from the femoral diaphysis of C57/Bl6 mice. Primary material and early passage cells were cultured in disposable plastic culture dishes (Sarstedt, Germany) under standard conditions. The accumulated cellular material was passaged to obtain the required number of cells for transplantation (Kladnytska et al., 2020). The obtained mesenchymal stem cells were analysed by flow cytometry, and the cells were incubated with PE Anti-Mouse CD90.2 (30-H12) antibody (Becton Dickinson, New Jersey, USA) at a dilution of 1:200, according to the manufacturer's instructions (Donnenberg et al., 2013). Data were analysed on a BD FACS Aria cell sorter (Becton Dickinson) using BD FACS Diva 6.1.2 software. A 488 nm laser and 585/42 nm filter were used (Robinson et al., 2023).

The mesenchymal stem cells of the red bone marrow culture of the 3rd passage were tested for viability. The viability of BM-derived aMSCs was determined by staining with trypan blue. A 0.4% aqueous solution of trypan blue (Sigma, USA) was added to the cell suspension, and the mixture was incubated for 2 min. Trypan blue stains dead cells or non-viable cells with damaged membranes blue. The resulting cell suspension was examined in a Goryaev chamber, and under low magnification, live (unstained) cells, as well as dead and damaged (blue) cells were visually counted.

Scheme of the experiment. Lewis lung carcinoma cells were transplanted into male C57/Bl6 mice in the thigh muscle area in the amount of 10⁴ cells in 0.5 cm³ of phosphate-buffered saline. From the moment of cancer initiation, C57/Bl6 mice were divided into three groups. The *first group* included mice with transplanted Lewis lung carcinoma; the *second group* included mice with transplanted Lewis lung carcinoma, which were injected with BM-derived AMSCs in the amount of 1.25×10⁴ on Day 8 after tumour cell transplantation. The *third group* included mice with transplanted Lewis lung carcinoma, which were injected with 0.89% NaCl solution (placebo effect).

The course of the tumour process of Lewis lung carcinoma (LLC) was characterised by standard indicators: weight of animals in the experimental groups, latent period of the primary tumour node, parameters of the primary tumour at different periods of tumour growth, the number of mice in the group with lung metastases, the average number of metastases per animal, tumour volume and infiltration of the primary tumour by lymphocytes were determined.

To isolate the mononuclear cell fraction, the method of separating cells of homogenized tumour tissue in a ficoll density gradient was used. The tumour tissue (2 g) from the experimental groups was homogenised. In a centrifuge tube, the tumour tissue homogenate was carefully layered onto ficoll ($\rho = 1.119$ g/mL) and centrifuged at 1,500 rpm. A layer of lymphocytes was collected over the entire cross-sectional area of the tube above the fraction of the ficoll solution, transferred to a clean centrifuge tube, added to Hanks' solution, and precipitated by centrifugation. The supernatant was removed, and the resulting precipitate was resuspended in a certain volume of Hanks' solution. The number of lymphocytes was counted in a Goryaev chamber.

Histological preparations were made to investigate the microscopic structure of muscle tissue at the site of Lewis lung carcinoma primary tumour localisation under the influence of BM-derived AMSCs. For this, fragments of skeletal muscles of the pelvic limb were separated with a sharp blade from the central and peripheral parts of the primary tumour node. Samples were kept for 5 days in a 10% aqueous solution of neutral formalin for fixation. The samples were then rinsed in running water and dehydrated in alcohols of increasing concentration, compacted, and embedded in paraffin on wooden blocks. Sections of skeletal muscle tissue 5-8 μm thick were made on a sled microtome MPS-2. Prepared sections were stained with Carazzi haematoxylin and eosin (Horal'skyi *et al.*, 2016). Histological specimens were used to investigate the structure of skeletal muscles at the site of her Lewis lung carcinoma. The area of the tumour parenchyma, vessels, tissue devitalisation areas, and necrotic lesions was calculated by the "point count" method using a Carl Zeiss 40 light-optical binocular microscope (Germany), a Weibel grid for measurements, a MBI-2 microscope and a MOV-1-15 $\times$ eyepiece micrometer, and UTHSCSA Image Tool software for Windows 7 (version 3.0).

The results were statistically analysed using ANOVA analysis of variance. The results are presented as $\bar{x} \pm \text{SD}$ (mean $\pm$ standard deviation). The reliability of the data obtained was assessed by the F-criterion, considering the Bonferroni correction, with a significance level of $P < 0.05$.

Results and Discussion

To conduct the experiment, prior to the study, work was done to obtain mesenchymal stem cells from red bone marrow culture for transplantation. According to the author's findings (Kladnytska *et al.*, 2020), red bone marrow aspirate from the femur and humerus of the limbs of C57/Bl6 mice served as the primary

material for obtaining cells with high proliferative activity. The red bone marrow aspirate obtained under sterile conditions was put into centrifuge tubes, phosphate-buffered saline was added and pipetted. Next, the cell suspension was precipitated by centrifugation at 300 g for 20 minutes. The supernatant was removed, and Dulbecco's modified Eagle medium (DMEM) was added to the cell pellet. The cells were suspended and placed in culture dishes for cultivation. The cell suspension was supplemented with 10-15% foetal bovine serum (FBS); 10 $\mu\text{L}/\text{cm}^3$ of antibiotic-antimycotic and cultured in a CO_2 incubator. Cells cultured *in vitro* have discrete properties compared to conventional cells and therefore require a suitable growth medium system. The nature of various environmental factors has a substantial impact on cells in culture. Most often, it is the type of substrate in which cells grow, the components of the culture medium, such as nutrients, chemical and physical properties of the medium, the use of biologically active substances (hormones and growth factors), the temperature of culture incubation, and the manipulations with cells that are provided for by the procedure of obtaining. Subculturing a culture can cause morphological and functional changes when the actual cultured cells change their shape, size, nuclear-cytoplasmic ratio, properties, and the use of such a graft can lead to false results.

The process of cultivation, preservation, and storage of stem cells involves the use of reagents that are not chemically neutral for biological objects. M. Awan *et al.* (2020) noted that dimethyl sulphoxide, which is used as a cryoprotectant to ensure the integrity of cell organelles, is toxic and affects the biological properties of the preserved material. Thus, stem cells obtained under different culture conditions, using additional factors to stimulate proliferation, differ in their biological and functional

properties, and in our opinion, they can have different effects in the recipient's body.

Thus, the choice of culture medium, the addition of growth factors for the cultured cells, and the culture conditions are crucial steps in maintaining cell proliferation and vital activity, as well as in preserving the actual morphology and function of the cultured cells. The first adherent cells appeared in the culture dishes after 24-48 hours of cultivation. On Day 3 of cultivation, the formation of mesenchymal stem cell colonies was recorded (Fig. 1).

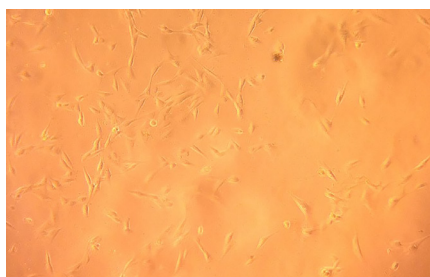


Figure 1. Colonies of mesenchymal stem cells of red bone marrow culture, Day 3 of cultivation

Notes: native preparation: red bone marrow culture stem cells, $\times 100$

Source: developed by the author of this study

As Figure 2 shows, mesenchymal stem cells adhered to the surface of the culture plastic are spread out over the surface and in contact with each other. Under these conditions, contact inhibition of cell proliferation begins, and therefore when the monolayer was 90-100% confluent, a 0.25% solution of trypsin and ethylenediaminetetraacetic acid (EDTA) was added to the culture and left in a thermostat at $+37^{\circ}\text{C}$ for 2 min.

Screening of red bone marrow culture MSC characteristics. Analysis of cytometry data revealed CD90 expression in 69% of cells in the 3rd passage. During the study of the viability of red bone marrow MSCs, it was found that 98%

of the cells did not pass the dye through the cytoplasmic membrane and did not stain blue, which confirms the integrity of the cell membrane and their viability. The findings obtained using the flow cytometry method confirmed that the red bone marrow MSC culture met the criteria for MSCs according to the recommendations of the International Society for Cell Therapy (Dominici *et al.*, 2006; Choudhery *et al.*, 2022). V.V. Kovpak & O.S. Kovpak (2018) emphasise that it is important to verify the quality and confirm the characteristics of the obtained cellular material, as this affects its proliferative and functional properties.

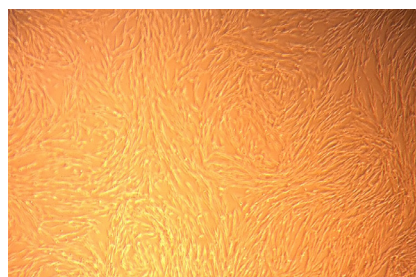


Figure 2. Mesenchymal stem cells of red bone marrow culture, Day 12 of cultivation, monolayer

Notes: native preparation: red bone marrow culture stem cells, $\times 100$

Source: developed by the author of this study

Mesenchymal stem cells are involved in numerous physiological processes, including organogenesis, tissue regeneration or repair, and maintenance of tissue homeostasis. G. Guo *et al.* (2021) recognise that MSCs play an important, dual, and complex role in cancer pathophysiology through their ability to limit or promote tumour progression. Specifically, in gastric cancer, mesenchymal stem cells inhibit the function of normal killer cells through signalling molecules and thus activate tumour progression.

According to C.R. Harrell *et al.* (2021), MSCs interact with the tumour microenvironment,

modulate tumour cell behaviour, affect their functions, and promote the formation of distant metastases through the secretion of mediators, modulation of the immune response, and regulation of intercellular interactions. This dynamic system is capable of inducing the formation of immunoprivileged tissue niches or the development of new tumours, which occurs as a result of the differentiation and proliferation of stem cells into tumour-associated fibroblasts and tumour stem cells. At the same time, MSCs have therapeutic effects, including anti-inflammatory, anti-tumour, antioxidant or antiproliferative effects. L. Frisbie *et al.* (2022) reported the ability of MSCs to selectively migrate and penetrate tumour sites. The interaction between tumour cells and non-malignant cells in the tumour microenvironment is a crucial factor in the course of the tumour process. Stromal, immune, endothelial cells, MSCs, which are involved in complex bidirectional cross-interaction with

malignant tumour cells, play a crucial role in the directed formation and functioning of the entire microenvironment of tumour tissue. Mesenchymal stem cells are reprogrammed by tumour cells into carcinoma-associated MSCs, which can differentiate into almost all stromal sublines, adipocytes, tumour-associated fibroblasts, and myofibroblasts. Carcinoma-associated mesenchymal stem cells and fibroblasts secrete most of the extracellular matrix in the tumour microenvironment and promote tumour growth and metastasis.

After transplantation of Lewis lung carcinoma cells into C57/Bl6 mice, the time of primary tumour formation in animals of all three groups was almost the same and amounted to 8.2 ± 0.40 days, 8.0 ± 0.35 days, and 8.2 ± 0.40 days, respectively. The body weight of mice with LLC under the influence of BM-AMSCs on Day 14 of the study significantly decreased by 13% ($P < 0.05$) compared to animals of the first and third groups (Table 1).

Table 1. Indicators of tumour diameter and body weight in experimental mice, $M \pm m$, $n = 9$

Tumour growth period, days	Group 1		Group 2		Group 3	
	body weight of mice, g	tumour diameter, mm	body weight of mice, g	tumour diameter, mm	body weight of mice, g	tumour diameter, mm
8	21.4 ± 0.4	4.8 ± 0.2	20.2 ± 0.7	5.0 ± 0.2	21.3 ± 0.4	4.9 ± 0.3
11	21.3 ± 0.5	5.3 ± 0.5	19.9 ± 0.6	$7.3 \pm 0.2^{*^{\wedge}}$	21.4 ± 0.3	5.2 ± 0.5
14	21.4 ± 0.2	6.2 ± 0.7	$18.7 \pm 0.6^{*^{\wedge}}$	$9.5 \pm 0.4^{*^{\wedge}}$	21.3 ± 0.5	6.3 ± 0.5
18	20.6 ± 0.8	8.1 ± 0.4	$18.1 \pm 0.4^{*^{\wedge}}$	$12.3 \pm 0.3^{*^{\wedge}}$	20.4 ± 0.6	8.2 ± 0.4
24	19.1 ± 0.4	8.9 ± 0.4	$17.7 \pm 0.3^{*^{\wedge}}$	$12.8 \pm 0.2^{*^{\wedge}}$	19.6 ± 0.4	8.5 ± 0.4

Notes: * – $P < 0.05$, ** – $P < 0.01$, *** – $P < 0.001$ relative to the data in the control group, ^ – $P < 0.05$, ^^ – $P < 0.01$, ^^ – $P < 0.001$ relative to the data in the placebo group

Source: developed by the author of this study

The body weight of mice in the experimental groups was inversely correlated with the size of the primary tumour node (Table 1). According to the findings, on Day 18 of the study, the tumour diameter increased 1.50 times in mice

under the influence of MSCs, and body weight decreased by 12.1% ($P < 0.05$) compared to the control group. Under the influence of MSCs, the diameter of the tumour in mice increased by 1.50 times, while the body weight of the

animals decreased by 11.3% ($P < 0.05$) compared to the placebo group.

Subsequently, on Day 24 of the experiment, the dynamics of changes in animal body weight and growth of the primary tumour node was maintained. Significant changes in body weight and diameter of the primary tumour node in mice under the influence of MSCs were recorded. According to the findings of the study, on Day 24 of the experiment, the tumour diameter in mice under the influence of MSCs increased by 1.43 times, and the body weight of the animals decreased by 7.3% ($P < 0.05$) compared to the animals of the control group. The tumour diameter in mice under the influence of MSCs increased by 1.51 times, while the body weight of the animals decreased by 9.7% ($P < 0.05$) compared to the placebo group. Indicators of animal body weight and tumour diameter of group 3 did not significantly differ from those of group 1, which demonstrates the absence of the effect of 0.89% NaCl solution on the promotion of LLC in the classical course.

Z. Jiang *et al.* (2023) noted that tumour-associated macrophages are the main inflammatory component of the stroma of many tumours, which can affect various aspects of neoplastic tissue. It is pericytes located between capillary endothelial cells and the basement membrane that perform the vasomotor function of capillaries, support tissue and organ microcirculation, induce macrophage polarisation, form a pre-metastatic niche, and are an essential component of the tumour microenvironment. S.S. Davidson *et al.* (2020), using RNA sequencing, investigated the diversity of tumour stromal cells at different stages of tumour development. Thus, stromal compartments were identified in murine melanoma and draining lymph nodes (LN) in the locations of tumour formation. Existing lymphocytes from the lymph nodes are activated and cloned within the tumour, while tumour-associated mye-

loid cells contribute to the formation of a suppressive niche. The authors of the publication emphasised the identification of three temporally distinct populations of stromal cells that demonstrated unique functional characteristics. According to their data, “immune” stromal cells predominate in the early stages of tumour development, and the population of “contractile” cells increases in the later stages of tumour progression. The C3 component of complement is specifically expressed in the immune population. It was found that its degradation product C3a engages C3aR+ macrophages, and changes in C3a and C3aR affect immune infiltration, inhibiting tumour development.

Numerous studies have shown that macrophages in the tumour microenvironment promote angiogenesis, matrix remodelling, and suppression of adaptive immunity, which at the final stage activates the tumour process. H. Kuroda *et al.* (2021) found a positive correlation between an increase in tumour-associated macrophages and a poor prognosis for patients. The researchers point out the significance of the tumour microenvironment. Specifically, an increase in the number of macrophages in the tumour stroma activates intercellular interaction and leads to activation of tumour growth.

Tumour-associated lymphocytes are involved in angiogenesis as producers of vascular endothelial growth factor (VEGF). An increase in the number of tumour-associated lymphocytes confirms the acceleration of the process of vascularisation of the primary tumour and, as a result, metastasis through the circulatory system. The obtained results show analogous data. The number of lymphocytes in the primary tumour tissue under the influence of red bone marrow-derived aMSCs significantly increased by 1.47 and 1.52 times on Day 18, respectively, compared to animals of the control group and placebo, respectively ($P < 0.05$).

Table 2. The number of lymphocytes in primary tumour tissue, $\times 10^6/\text{g}$ tumour tissue, M + m, n = 9

Tumour growth period, days	Group of mice		
	1	2	3
Day 18	1.83 $\pm$ 0.35	2.72 $\pm$ 0.11 ^{*^}	1.79 $\pm$ 0.29
Day 24	2.13 $\pm$ 0.23	2.83 $\pm$ 0.35 ^{*^}	2.11 $\pm$ 0.15

Notes: * – $P < 0.05$, ** – $P < 0.01$, *** – $P < 0.001$ relative to the data in the control group, ^ – $P < 0.05$, ^^ – $P < 0.01$, ^^ – $P < 0.001$ relative to the data in the placebo group

Source: developed by the author of this study

Comparable results were obtained on Day 24 of the development of the primary tumour node of Lewis lung carcinoma. The number of lymphocytes in the primary tumour tissue under the influence of red bone marrow-derived aMSCs on day 24 significantly increased by 1.33 and 1.34 times compared to control and placebo animals, respectively ($P < 0.05$). This suggests that MSCs create the conditions for vascularisation of the tumour node, and as a result, metastasis by haematogenous means, and, accordingly, activation of the tumour process.

These results are in line with the findings of a study on primary tumour metastasis to the lungs. Notably, in mice of group 2 with LLC, under the influence of red bone marrow-derived aMSCs, the process of lung metastasis was accelerated. This is confirmed by a significantly larger volume of lung metastases, which was $41.52 \pm 7.9 \text{ mm}^3$ ($P < 0.05$) compared with the indicators of animals of groups 1 and 3, where they were 17.94 ± 6.59 and $16.43 \pm 5.32 \text{ mm}^3$, respectively. Thus, in mice with Lewis lung carcinoma, transplantation of red bone marrow-derived aMSCs significantly affected the process of metastasis and promoted the active transition of lung metastases to the vascular stage.

It was found that MSCs demonstrate high plasticity and undergo functional changes in different microenvironments. Mesenchymal stromal cells are considered to be one of the important components of the tumour microenvironment that induce tumour development. Y. Miyazaki *et al.* (2021) found that

MSCs do not inherently promote tumour development, but they acquire tumourigenic properties when exposed to components of the tumour microenvironment. In this context, various elements of the tumour microenvironment that affect the biological characteristics of MSCs through complex interactions are considered: tumour cells, pro-inflammatory factors, immune cells, extracellular matrix, and hypoxia. The researchers noted that in pancreatic ductal adenocarcinoma, cancer-associated fibroblasts are a key component of the proliferating tumour stroma. It was found that adipose tissue culture stem cells can differentiate into separate subtypes of cancer-associated fibroblasts depending on the culture conditions *in vitro*. Therefore, the identification of potential markers of cancer-associated fibroblast subtypes will enable the investigation of the mechanisms of the latter's influence on tumorigenesis.

J. Liu *et al.* (2020) summarised the data on the multifaceted therapeutic functions of MSCs, but in general, they are able to balance the regenerative and inflammatory microenvironment of the affected tissue in the case of severe inflammation. The interaction between MSCs and the immune system was demonstrated by changes in the activity of B lymphocytes. MSCs can induce the formation of regulatory B cells through intercellular contact, soluble factors, and extracellular vesicles. Thus, these cells can complement each other's immunomodulatory functions.

H. Gu *et al.* (2022) noted that extracellular vesicles have a substantial impact on MSCs in the tumour microenvironment, as they carry a variable range of molecules, such as RNA, and can therefore induce reprogramming of MSCs by exosomal oncogenic factors. Exosomes derived from MSCs from various sources, including adipose tissue, bone marrow, umbilical cord, and human olfactory bulbs, showed inhibition of proliferation, migration, and invasion of colorectal cancer cells through regulation of the RAP2B/PI3K/AKT and ITGA2/ITGA6 signalling pathways.

J. Kim *et al.* (2021) investigated methods for obtaining highly purified exosomes from human umbilical cord MSCs and bovine foetal serum based on ultracentrifugation and a tangential flow filtration system. Exosomes derived from human umbilical cord MSCs based on the tangential flow method demonstrated a high angiogenic effect and faster wound healing by 71.4% and 23.1%, respectively.

P. Bule *et al.* (2021) showed that MSCs are exposed to components of the tumour microenvironment and can induce angiogenesis through numerous proangiogenic factors. It was found that after treatment of MSCs with conditioned medium collected from tumour cells, the amount of proangiogenic factors in the tumour microenvironment (mRNA of TGF- β , VEGF, IL-6 and MIP-2) increased. The authors noted that chemokines – chemotactic cytokines – coordinate the exchange between cells in the tumour microenvironment, affect metastasis, angiogenesis, cancer cell proliferation, invasiveness and stemness, and therefore are key dominants in the development of the disease. Many chemokine receptor inhibitors targeting various chemokine signalling pathways are currently being evaluated to modulate the tumour microenvironment and optimise the immune response to overcome patient resistance to chemotherapy. A. Birbrair (2020)

reported that MSCs, when interacting with the tumour microenvironment, can directly promote the growth and migration of tumour cells, suppress anti-tumour immunity, and contribute to angiogenesis and overall tumour promotion. Thus, the data presented in this paper on the involvement of MSCs in tumour vascularisation both *in vivo* and *in vitro* coincide with the findings of the above studies.

However, there are well-known scientific studies by H. Gu *et al.* (2021), which proved that MSCs can effectively inhibit tumour progression. The effect of exosomes from MSCs on hepatocellular carcinoma stem cells *in vitro* was investigated. As a result, it was proved that exosomes derived from MSCs block the “malignant behaviour” of hepatocellular carcinoma stem cells due to a considerable reduction in their proliferation, migration, invasion, stimulation of angiogenesis, and self-renewal. This suggests the need to consider the conditions of the experiment. Under these conditions, the MSCs were not exposed to the tumour microenvironment, because the duration of co-cultivation of MSCs with carcinoma cells does not provide the interaction of microenvironmental factors that occurs *in vivo*. *In vivo* hypoxia conditions in tissues also have an impact on tumour development.

S. Bagheri-Mohammadi *et al.* (2020) performed the application of human adipose tissue culture MSCs transfected with TSP-1 factor as a vector therapy for melanoma in mice. As noted above, MSCs affect the tumour process due to their localisation in the microenvironment of tumour tissue. When allogeneic MSCs were injected into bone marrow cultures of animals with Lewis lung carcinoma, the microscopic structure of muscle tissue was noticeably different from that of the control group at all periods of observation.

Under the influence of bone marrow culture MSCs on Day 18 of the study in C57Bl6

mice, the progression of tumour growth in skeletal muscles was found to be greater compared to its spontaneous development. The histological sections revealed that the primary tumour of Lewis lung carcinoma consisted of polymorphic round and oval cells. A nucleus with a large nucleolus occupied almost the entire cell and contained a nucleolus (Fig. 3).

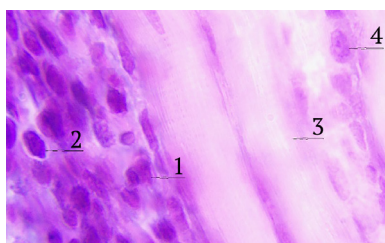


Figure 3. Skeletal muscle of the mouse during the development of primary Lewis lung carcinoma tumour using allogeneic MSCs of red bone marrow culture, Day 18 of the study

Notes: Histological preparation: 1 – nucleus occupying the entire cell with a large nucleolus; 2 – oval-shaped LLC cells; 3 – longitudinal section of skeletal muscle; 4 – daughter cell of the primary tumour growth. Carazzi hematoxylin and eosin staining, $\times 400$

Source: developed by the author of this study

The primary tumour tissue is formed by atypical cells of predominantly round and oval shapes with intensely basophilic large nuclei. The tumour did not have a distinct stroma, which confirms a high degree of its anaplasia. The nuclei of the cells are large, the vast majority of them have a distinct nucleus. The cytoplasm of all tumour cells was intensively stained with Carazzi haematoxylin, which confirms active processes of RNA and, accordingly, protein synthesis. Single giant multinucleated tumour cells were detected. There were signs of metastasis by extension and hematogenous metastasis. These signs are typical for the most malignant tumours and indicate active growth of tumour tissue and progression of the process. On histological sections on Day 18 of the

study, under the influence of BM-AMSCs, active metastasis by extension was recorded: the penetration of LLC cells between muscle fibres from the primary tumour node, which formed daughter metastases, was recorded (Fig. 3). There were also relatively large foci of haemorrhage and necrosis of tumour tissue, which were mainly localised in the central part of the tumour, but sometimes also in the peripheral areas (Figs. 4, 5).

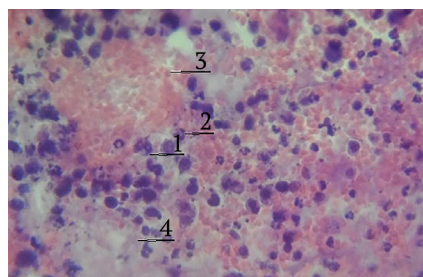


Figure 4. Skeletal muscle of the mouse during the development of primary Lewis lung carcinoma tumour using allogeneic MSCs of red bone marrow culture, Day 18 of the study

Notes: Histological preparation: 1 – mitosis; 2 – light areas of the nucleus – chromatin; 3 – haemorrhage; 4 – tissue necrosis. Carazzi haematoxylin and eosin staining, $\times 200$

Source: developed by the author of this study

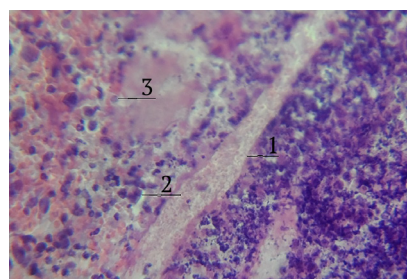


Figure 5. Skeletal muscle of the mouse during the development of primary Lewis lung carcinoma tumour using allogeneic MSCs of red bone marrow culture, Day 18 of the study

Notes: Histological preparation: 1 – blood vessel; 2 – multinucleated cell; 3 – karyolysis. Carazzi haematoxylin and eosin staining, $\times 200$

Source: developed by the author of this study

Notably, on Day 18 of the study, the density of blood vessels in the tumour tissue under the influence of BM-derived aMSCs was 1.4 times higher than in the control and placebo groups. The specific gravity of parenchyma decreased compared to the control group and was $65.5 \pm 2.3\%$ ($P < 0.05$). The specific gravity of necrosis areas was almost 2 times higher than that of the control group and amounted to 25.7 ± 4.2 ($P < 0.05$). The studied morphological features of LLC tumour tissue are fully confirmed by the data obtained on the progression of the primary tumour, activation of the lung metastasis under the influence of BM-AMSCs. Studies have shown that MSCs can stimulate the growth of cancer cells and angiogenesis of the primary tumour. In breast and prostate tumours, according to T.E. Krueger *et al.* (2019), MSCs also increased the amount of proangiogenic factors such as VEGF, MIP-2, IL-6, and TGF- β . These factors, as noted by F. Ah-Pine *et al.* (2023), induced tumour cell proliferation and angiogenesis, which substantially accelerated the rate of tumour development *in vitro* and *in vivo*.

Thus, the progression of tumour growth depends entirely on multidirectional signals of soluble factors and intercellular interaction of the tumour microenvironment. Allogeneic MSCs are actively embedded in the tumour stroma, produce exosomes containing many gene products that have an ambiguous effect on a wide range of cells, their phenotype, biological function, induce active cellular responses, and create conditions for nanoconnection of tumour microenvironment components. As a result of this interaction, we observed progression of tumour growth, active vascularisation of tumour tissue, acceleration of metastasis, and its transition to the vascular stage.

Conclusions

The use of allogeneic mesenchymal stem cells from bone marrow culture in C57BL6 mice with

Lewis epidermoid metastatic lung carcinoma promoted the activation of the tumour process. After transplantation of Lewis lung carcinoma cells, the period of primary tumour formation in animals of all three groups was practically the same and was 8.2 ± 0.40 days, 8.0 ± 0.35 days, and 8.2 ± 0.40 days, respectively. Under the influence of allogeneic MSCs of red bone marrow culture from Day 14 to Day 24 of the study, the weight of mice decreased by 7.0-12.1% ($P < 0.05$) compared to the control, the diameter of the primary tumour increased by 1.43-1.51 times ($P < 0.05$), which is associated with the activation of primary tumour growth. On Day 18 of the experiment, the number of tumour-associated lymphocytes increased by 1.47 and 1.52 times compared to the control and placebo groups, respectively ($P < 0.05$). Analogous results were obtained on Day 24 of growth of the primary Lewis lung carcinoma tumour. This suggests that MSCs create the conditions for accelerating the vascularisation of the primary tumour and, as a result, metastasis through the circulatory system. Under the influence allogeneic mesenchymal stem cells of bone marrow culture in mice, the process of lung metastasis was accelerated. This is confirmed by a significantly larger volume of lung metastases, which was 1.52 ± 7.9 ($P < 0.05$) compared with the animals of the first and third groups, in which these indicators were $17.94 \pm 6.59 \text{ mm}^3$ and $16.43 \pm 5.32 \text{ mm}^3$, respectively. The morphological picture of the histological sections of the primary tumour of Lewis lung carcinoma confirms all the signs of qualitative and quantitative indicators of its progression. Under the influence of bone marrow culture MSCs on Day 18 of the study in C57BL6 mice, the progression of the primary tumour in skeletal muscle tissue was found compared to its spontaneous development. The histological sections revealed that the primary tumour of Lewis lung carcinoma consisted of polymorphic round and oval cells

with an intensely basophilic large nucleus and a nucleolus that occupied almost the entire cell. The cytoplasm of all tumour cells was intensively stained with Carazzi haematoxylin, which confirms active processes of RNA and, accordingly, protein synthesis. Single giant multinucleated tumour cells were detected. The tumour did not have a distinct stroma, which confirms a high degree of its anaplasia. There were signs of metastasis by extension and hematogenous metastasis. Relatively large foci of haemorrhage and necrosis of tumour tissue were detected, which were mainly localised in its cen-

tral part. These signs are typical for the most malignant tumours and indicate active growth of tumour tissue and progression of the process.

In the future, it is planned to determine the influence of allogeneic stem cells from the culture of adipose and nervous tissue on the tumor process.

Acknowledgements

None.

Conflict of Interest

None.

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Вплив алогенних мезенхімних стовбурових клітин культури червоного кісткового мозку на розвиток епідермоїдної карциноми легені Льюїс *in vivo*

Лариса Кладницька

Доктор ветеринарних наук, професор
Національний університет біоресурсів і природокористування України
03041, вул. Героїв Оборони, 15, м. Київ, Україна
<https://orcid.org/0000-0002-9360-0587>

Віктор Томчук

Доктор ветеринарних наук, професор
Національний університет біоресурсів і природокористування України
03041, вул. Героїв Оборони, 15, м. Київ, Україна
<https://orcid.org/0000-0003-0622-6345>

Владислав Величко

Аспірант
Національний університет біоресурсів і природокористування України
03041, вул. Героїв Оборони, 15, м. Київ, Україна
<https://orcid.org/0000-0002-5227-9168>

Володимир Салата

Доктор ветеринарних наук, професор
Львівський національний університет ветеринарної медицини та біотехнологій
імені С. З. Гжицького
79010, вул. Пекарська, 50, м. Львів, Україна
<https://orcid.org/0000-0002-7175-493X>

Яков Шенгаут

Кандидат ветеринарних наук
Ветеринарний центр Якова Шенгаута
03147, вул. Геросіос Вільтієс, 1, м. Вільнюс, Литва
<https://orcid.org/0000-0002-5471-0046>

Анотація. Актуальність дослідження зумовлена широким використанням стовбурових клітин у ветеринарії та медицині, великим спектром досліджень та неоднозначними даними щодо онкопротекторних властивостей стовбурових клітин різного походження. А тому, мета представленої наукової роботи стосувалася дослідження перебігу пухлинного процесу за карциноми легені Льюїс та особливостей впливу на нього алогенних мезенхімних стовбурових клітин культури червоного кісткового мозку. Провідним підходом дослідження цієї проблеми є метод моделювання карциноми легені Льюїс в мишей C57BL6

та застосування при цьому стовбурових клітин. Використання аlogenних мезенхімних стовбурових клітин культури кісткового мозку мишей C57BL6 з трансплантованою епідерміоїдною метастатичною карциномою легені Льюїс сприяло активізації пухлинного процесу. За впливу аlogenних мезенхімних стовбурових клітин культури червоного кісткового мозку з 14 до 24 доби дослідження вага мишей зменшувалася на 7,0-12,1 % ($P < 0,05$) відносно контролю, діаметр первинної пухлини збільшувався в 1,43-1,51 раза ($P < 0,05$), що обумовлено активізацією росту первинної пухлини. Кількість лімфоцитів, як продуцентів фактору росту судин у первинній пухлинній тканині, за впливу аlogenних мезенхімних стовбурових клітин культури червоного кісткового мозку достовірно зростала вже на 18 добу експерименту в 1,47 і 1,52 раза порівняно з тваринами контрольної групи та плацебо ($P < 0,05$), відповідно. Це сприяло ангіогенезу в первинному пухлинному вузлі та метастазуванню через систему кровообігу. За введення мишам аlogenних мезенхімних клітин культури червоного кісткового мозку зареєстровано більший об'єм метастазів у легені, який становив $41,52 \pm 7,9$ мм³ порівняно з показниками в тварин контрольної групи та плацебо, відповідно $17,94 \pm 6,59$ і $16,43 \pm 5,32$ мм³. Морфологічна картина гістозрізів первинної пухлини карциноми легені Льюїс підтверджує всі ознаки якісних і кількісних показників її прогресії. Отримані результати становлять як теоретичну, так і практичну цінність для клінічної ветеринарної медицини з питання застосування аlogenних мезенхімних стовбурових клітин кісткового мозку за пухлинного процесу

Ключові слова: первинна пухлина; інфільтрація; лімфоцити; метастази; васкуляризація; миші C57BL6



2024. Volume 15, No. 2. P. 121-137

Received: 26.01.2024 Revised: 24.04.2024 Accepted: 29.05.2024

DOI: 10.31548/veterinary2.2024.121

UDC 636.9: 591.48:591.111.1

Influence of autonomic nervous system tone on the content of unsaturated fatty acids in blood lipids in goats

Bożena Króliczewska*

PhD in Technical Sciences, Associate Professor
Wrocław University of Environmental and Life Sciences
50375, 31 C.K. Norwida Str., Wrocław, Poland
<https://orcid.org/0000-0002-0291-3562>

Bohdan Boychuk

Postgraduate Student
National University of Life and Environmental Sciences of Ukraine
03041, 15 Heroiv Oborony Str., Kyiv, Ukraine
<https://orcid.org/0009-0000-6169-004X>

Valentin Karpovskyi

Doctor of Veterinary Sciences, Professor
National University of Life and Environmental Sciences of Ukraine
03041, 15 Heroiv Oborony Str., Kyiv, Ukraine
<https://orcid.org/0000-0003-3858-0111>

Ihor Hryshchuk

Postgraduate Student
National University of Life and Environmental Sciences of Ukraine
03041, 15 Heroiv Oborony Str., Kyiv, Ukraine
<https://orcid.org/0000-0003-2571-6876>

Andriy Hryshchuk

PhD in Veterinary Sciences, Associate Professor
Luhansk Taras Shevchenko National University
36003, 3 Kovalya Str., Poltava, Ukraine
<https://orcid.org/0000-0002-4608-337X>

Abstract. The relevance of this study lies in the need to determine the role of the autonomic nervous system in the metabolism of unsaturated fatty acids, especially essential ones, in the

Suggested Citation:

Króliczewska, B., Boychuk, B., Karpovskyia, V., Hryshchuk, I., & Hryshchuk, A. (2024). Influence of autonomic nervous system tone on the content of unsaturated fatty acids in blood lipids in goats. *Ukrainian Journal of Veterinary Sciences*, 15(2), 121-137. doi: 10.31548/veterinary2.2024.121.

*Corresponding author



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body of goats, which will improve the understanding of individual metabolic characteristics in these animals. The use of established blood markers will make it possible to analyse the state of lipid metabolism in the body of goats and ensure an increase in the effectiveness of methods of its correction. The purpose of this study was to determine the specific features of the autonomic nervous system influence on the relative content of unsaturated fatty acids in goat plasma lipids. The experiment involved goats of the Zaanenska breed, of which, using electrocardiographic analysis according to the Baevsky method and depending on the tone of the autonomic nervous system, three experimental groups were formed: normotonics, sympathotonics, and vagotonics. The method of gas-liquid chromatography was used to determine the percentage of unsaturated fatty acids in goat plasma lipids. Thus, in goats with sympathotonics, which have a predominance of sympathetic nervous system activity, a high percentage of the following lipids in blood plasma was noted: linolenic ($P < 0.01$), cis-4, 7, 10, 13, 16, 19-docosahexaenoic ($P < 0.05$), docosahexapentaenoic ($P < 0.001$), and arachidonic acids ($P < 0.001$) compared to normotonics. These animals also had a lower content of palmitoleic ($P < 0.05$) and oleic acids ($P < 0.01$) in plasma lipids against the background of normotonics. In vagotonic goats, in which the influence of the parasympathetic nervous system predominates, the relative content of cis-4, 7, 10, 13, 16, 19-docosahexaenoic ($P < 0.05$), linoleic ($P < 0.001$) and arachidonic acids ($P < 0.001$) and high cis-11-eicosenoic acid ($P < 0.01$) in blood plasma lipids was found to be low compared to normotonic goats. The established features suggest a substantial influence of the tone of the autonomic nervous system on the quantitative redistribution of unsaturated fatty acids in the composition of goat blood plasma lipids and the need to consider them when analysing the state of the corresponding metabolism in the body, which will substantially improve not only the effectiveness of scientific research, but also its reliability

Keywords: sympathotonia; vagotonia; normotonia; gas-liquid chromatography; plasma

Introduction

Globally, dairy products form an integral part of the diet of most people. In terms of global consumption, cow's milk is the leader, followed by buffalo, and goat's milk, which is rapidly gaining momentum (Monllor *et al.*, 2020). According to A. Maslyuk *et al.* (2020), goat breeding is rapidly beginning to catch up with previous favourites in consumer choice. This can be explained by the fact that people are increasingly faced with the impossibility of consuming cow's milk due to the loss of its full absorption or the development of an allergic reaction. In such situations, goat's milk is a good alternative for consumers. The biological value of goat products is not inferior to that of dairy cattle, and in some

respects is even better. According to H. Sedilo *et al.* (2020), this contributes to the intensive development of goat milk production enterprises.

As a result, farm owners are faced with the question of how to improve milk quality at a lower economic cost. The question of the need to enrich milk with essential fatty acids is often raised. Products with a high content of these components, namely omega 3, omega 6, and omega 9 fatty acids, improve the metabolism of mammals, especially lipids. According to J. Djordjevic *et al.* (2019), considering the quality indicators of dairy products, one of the most key factors for evaluation is the study of their fatty acid content, specifically, unsaturated

fatty acids. Today, scientists are actively investigating the issues related to increasing the content of essential fatty acids in dairy products, which creates different avenues for solving them. For this, the introduction of unsaturated fatty acids into the body of goats with the help of various plant-based additives is widely used. Therewith, there were slight changes in the content of fatty acids in the blood against the background of substantial disorders of scarred digestion. Few people pay attention to the fact that not only the diet consumed by the animal will play a role in the concentration of unsaturated fatty acids in the blood and milk. N. Ghavipanje *et al.* (2022) believe that metabolic processes in the body itself and systems that substantially affect homeostasis in general should be considered. This implies the significant role of the autonomic nervous system, which forms an integral part of the centralised system that regulates the synthesis and breakdown of nutrients.

The study of the role of the autonomic nervous system tone in goats, which is a characteristic of the specific individual features of the animal depending on the predominance of the influence of the sympathetic or parasympathetic nervous system, will be a key factor in the analysis of the content of fatty acids in blood plasma lipids. Therefore, depending on the sympatho-vagal balance – normotonia, vagotonia, sympathotonia – the synthesis and breakdown of nutrients in the animal body will differ. Accordingly, the processes of digestion and absorption of nutrients in the gastrointestinal tract will proceed at different rates. Notably, the use of energy-rich substances by the body's cells with the corresponding metabolic rate will also differ. This should be considered when calculating the diet for goats, but generally, the general schemes for highly productive animals should be followed. As a result, different indicators are noted in terms of both milk

fat content and productivity of these animals. A.K. Verma *et al.* (2023) argued that it is necessary to increase the dietary intake of essential fatty acids in goats to enrich their dairy products. Consideration of new facts in the investigation of lipid homeostasis in goats depending on the sympathetic-vagal balance will be the basis for ensuring the reliability of scientific results. As a result, I. Erez & U. Serbester (2023) noted that scientists in research on improving the content of unsaturated fatty acids in goat dairy products need to have additional markers for effective statistical analysis of the results.

Therefore, the purpose of this study was to determine the specific features of the quantitative redistribution of unsaturated fatty acids in blood plasma lipids depending on the sympathetic-vagal balance in the body of clinically healthy goats.

Literature Review

Over the past 50 years, dairy production from goats has increased to 53% and continues to grow. Among the countries most actively developing in this area, Asia ranks first, followed by Africa, Europe, and the Americas (Nudda *et al.*, 2021). The most common products made from goat's milk are yoghurts, cottage cheese, and ryazhanka. A. Mavrommatis *et al.* (2020) noted that goat's milk is a good source of fat and protein. The farms that supply these products are committed to improving the quality of their raw materials. A. Lopez *et al.* (2019) noted that the key indicator in this area is the fatty acid content. Comparing milk from cows and goats, the ratio of saturated and unsaturated fatty acids differs. According to S. Mitsiopoulou *et al.* (2021) and X. Tian *et al.* (2022), goat products are healthier because they contain a higher amount of polyunsaturated fatty acids, which ensure the endogenous synthesis of biologically active substances and prevent the accumulation of excess cholesterol in the body.

P. Monllor *et al.* (2020) developed an entire scheme of balanced diets with different composition of plant feeds for goats to improve the intake and synthesis of unsaturated fatty acids. In addition, various mixtures are added to the animals' diet in the form of compound feed, which includes corn, rapeseed, flax, sesame seeds, etc. The results of using this additional supplement changed the fatty acid composition of milk. A. Nudda *et al.* (2021) noted an increase in the content of unsaturated fatty acids, especially polyunsaturated ones, which further increases the oxidative stability of products. However, not all methods involving dietary changes improved the ratio of fatty acids; in most cases, the changes were insubstantial. When using diets enriched with unsaturated fatty acids, the researchers also noted the consequences.

One of the most common problems faced by researchers is the disruption of scar digestion in ruminants. The activity of the microflora in the stomach is highly dependent on the environment. Specifically, excessive intake of unsaturated fatty acids in the stomach can have negative consequences. Thus, rumen microorganisms are activated to provide biohydrogenation of linoleic acid to reduce unsaturated fatty acids and increase saturated fatty acid content. Such processes are explained by the fact that polyunsaturated fatty acids in excess can cause changes in the physical and chemical properties of bacterial cell membranes and provoke their destruction. According to A. Nudda *et al.* (2021), this not only reduces the number of microorganisms in the rumen, but also increases the content of saturated fatty acids.

Notably, the mechanisms of ensuring the homeostasis of lipid metabolism in goats have individual characteristics. As stated by J. Orzuna-Orzuna *et al.* (2023), many factors affect the transformation of lipids in tissues, which provides the necessary conditions for the synthesis and decomposition of the relevant substances.

Goats are constantly exposed to a variety of factors that can stimulate or inhibit biochemical processes. A classic example of a mechanism for changing the steady course of any type of metabolism is the development of stress. This is the most common reason that every animal faces in its life, and depending on its individual characteristics, adaptation to it occurs in different ways. B. Paszczyk *et al.* (2020) note that the consequences of this process are differences in the absorption and use of the same lipids that have the highest energy value.

H. Tian *et al.* (2022) pointed out that lipid metabolism and the concentration of these substances in the blood are affected by diet, conditions of detention, the functional state of body systems, especially the digestive system, etc. This study discusses the role of the autonomic nervous system in these processes. As noted by Jr. Myers *et al.* (2022), the sympathetic and parasympathetic parts of the nervous system, through their coordinated influence on the body, correct metabolic processes. The sympathetic nervous system contributes to the body's adaptation to a stressful factor by increasing the activity of the cardiovascular system, enhancing the processes of nutrient breakdown, etc. The parasympathetic system has the opposite function – it slows down the activity of the cardiovascular system, activates digestion, and endogenous synthesis of substances important for the body. These systems work in concert to ensure that the body adapts to endogenous changes and changing environmental conditions. At the same time, each animal has specific individual features in terms of the functioning of adaptation mechanisms. This implies a significant regulatory role for the tone of the autonomic nervous system, which, depending on the predominance of the sympathetic and parasympathetic nervous system, can divide all animals into three groups: normotonics, sympathotonics, and vagotonics. According to

D. Goldstein (2020) and A. Lima *et al.* (2022), the specific features of the influence of the autonomic nervous system tone should be considered when analysing homeostasis indicators.

Materials and Methods

The material for the study was collected from goats of the Zaanenska breed based on a private dairy farm in the village of Kniahynynok, Lutsk district, Volyn region, in 2022-2023. For the study, animals of the same age were pre-selected, and a clinical examination was carried out to confirm the health status of 60 goats. The next stage was the formation of research groups from the selected livestock that met the criteria for the experiment.

Experimental groups of animals were formed by means of a variational pulse oximetry study according to the method of R.M. Bae-vsky *et al.* (2001). The goats were divided into three experimental groups of five each according to the tone of autonomic nervous regulation: sympathotonics, vagotonics, and normotonics. This division was based on an electrocardiological study considering the following indicators: mode (Mo), mode amplitude (AMo), variation range (Δx), autonomic rhythm index (ARI), autonomic balance index (ABI), and voltage index (VI) (Bae-vsky *et al.*, 2001). According to the results of the variational pulse study, it was found that normotonics have a balanced influence of the sympathetic and parasympathetic nervous systems; vagotonics have a predominant influence of the parasympathetic nervous system; and sympathotonics have a predominant influence of the sympathetic nervous system. As a result, three experimental groups of animals were formed with different tones of the autonomic nervous system, reflecting the individual characteristics of their organism.

Blood was collected in the morning 4 hours after feeding from the jugular vein using a sterile heparin syringe at the rate of 3 drops of 1%

heparin solution per 10 mL of blood. The samples were transported in a thermal container at +4°C. To obtain plasma, blood with anticoagulant was centrifuged at 2,000 rpm for 10 min. Lipid extraction from goat plasma was performed according to the method of J. Folch *et al.* (1957). Blood plasma was introduced into a flask with a lapped lid, then a mixture of methanol and chloroform was added to it in a ratio of 2:1 and to the test material in a ratio of 1:20. Subsequently, the flask was closed and the contents were shaken thoroughly, leaving them overnight for extraction. The next day, the mixture was filtered and 0.74% KCl was added to the filtrate in such a concentration that its content corresponded to one fifth of the total volume. Then it was settled overnight to separate the mixture into two phases. The lower phase was taken off and chloroform was extracted using an extractor, while the lipid residue was used for chromatographic analysis (Folch *et al.*, 1957).

The chromatographic study was performed using a Trace GC Ultra chromatograph (USA) with a flame ionisation detector. Chromatography of the samples was performed at the following parameters: detector temperature +260°C, column temperature +140-240°C. A 1 μ L sample was injected into the chromatograph using a TriPlus autosampler. The analysis time for one test sample was 65 min. A standard sample of Supelco 37 Component FAME Mix (USA) was used to identify the results of a chromatographic study to determine the fatty acid composition of plasma lipids in goats. The study was conducted in three parallel groups. The analysis of the chromatographic display of the components of the material under study was determined considering the following characteristics: peak retention time, peak base, peak area, peak height, peak width, and peak half-width. The chromatograph software automatically processes the results in the following steps: noise filtering, peak marking, qualitative

analysis with peak identification, and quantitative analysis of the area or height of the peaks. The results obtained during the distribution of the extracted lipids in the chromatograph column were compared with the standard for the identification of fatty acids in the sample.

Procedures for sampling biological material from goats for subsequent fatty acid analysis of plasma lipids were carried out following ARRIVE recommendations without violating the main provisions of EU Directive 2010/63/EU "On the Protection of Animals Used for Scientific Purposes" (2010).

The statistical processing of the study results involved the use of Microsoft Excel with an assessment of the significance of the differ-

ence in assessment indicators according to Student's t-test and $P < 0.05$, $P < 0.01$, $P < 0.001$.

Results and Discussion

The statistical analysis of the chromatographic results revealed differences in the content of unsaturated fatty acids depending on the tone of autonomic nervous regulation (Table 1). The relative content of omega-3, omega-6, and omega-9 acids in goat plasma lipids was determined among the essential fatty acids. Animals with different tone of the autonomic nervous system – normotonics, vagotonics, and sympathotonics – differed in the relative content of unsaturated fatty acids compared to that of the experimental group (normotonics).

Table 1. Content of unsaturated fatty acids in plasma lipids in goats depending on the tone of autonomic nervous regulation ($M \pm m$; $n = 5$, %)

Unsaturated fatty acid	Normotonics	Sympathotonics	Vagotonics
Myristoleic C14:1	0.18 ± 0.01	0.15 ± 0.01	$0.23 \pm 0.01^{**}$
Palmitoleic C16:1 ω 9	0.44 ± 0.03	$0.34 \pm 0.02^*$	0.51 ± 0.04
Oleic C18:1 ω 9	18.89 ± 0.14	$18.02 \pm 0.20^{**}$	$18.46 \pm 0.10^*$
Linoleic C18:2 ω 6	24.54 ± 0.51	24.65 ± 0.43	$20.69 \pm 0.19^{***}$
Linolenic C18:3 ω 3	1.04 ± 0.06	$1.33 \pm 0.05^{**}$	1.03 ± 0.05
Cis-11-eicosenoic, C20:1 ω 9	0.47 ± 0.01	$0.51 \pm 0.01^*$	$0.53 \pm 0.01^{**}$
Cis-8,11,14-eicosatrienoic C20:3 ω 6	0.07 ± 0.01	$0.11 \pm 0.01^*$	$0.15 \pm 0.01^{***}$
Arachidonic C20:4 ω 6	6.75 ± 0.14	$7.75 \pm 0.15^{***}$	$5.58 \pm 0.11^{***}$
Docosapentaenoic C22:5 ω 3	0.22 ± 0.01	$0.34 \pm 0.01^{***}$	0.24 ± 0.01
Cis-4,7,10,13,16,19-docosaheptaenoic C22:6 ω 3	0.76 ± 0.05	$0.94 \pm 0.04^*$	$0.64 \pm 0.01^*$

Notes: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ – relative to the data in the experimental group (normotonic)

Source: developed by the author of this study

Analysing the relative content of unsaturated fatty acids in goat plasma lipids, it was found that the most significant total volume is made up of such acids as oleic, linoleic, linolenic, arachidonic, and cis-4, 7, 10, 13, 16, 19-docosaheptaenoic. The highest content of omega-3 fatty acids was recorded in sympathotonics. Thus, in sympathotonics, the level of linolenic acid in the blood plasma is 1.3 times higher than in normotonic goats, which is $1.33 \pm 0.05\%$ ($P < 0.01$). Furthermore, the highest

percentage of sympathotonics was observed for docosapentaenoic acid (1.6 times), which was $0.34 \pm 0.01\%$ ($P < 0.001$). The content of cis-4, 7, 10, 13, 16, 19-docosaheptaenoic acid was 1.2 times higher in goats with sympathotonia $0.94 \pm 0.04\%$ ($P < 0.05$) compared to normotonics and 1.5 times higher in vagotonics $0.64 \pm 0.01\%$ ($P < 0.05$). Thus, animals with a predominance of sympathetic nervous system processes had the highest levels of unsaturated fatty acids belonging to the omega-3 fatty acid family (Fig. 1).

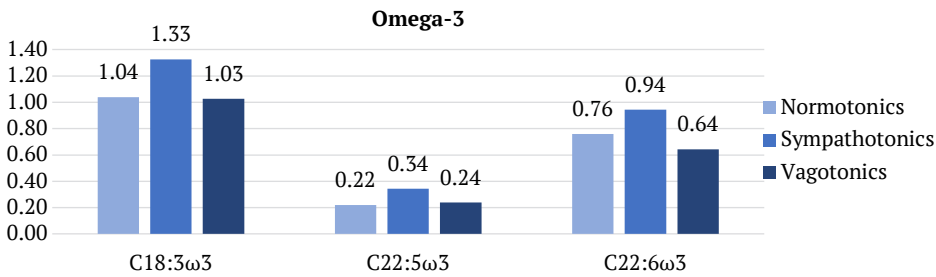


Figure 1. The content of omega-3 fatty acids (%) in blood plasma lipids in goats of experimental groups

Source: developed by the author of this study

The high content of omega-3 fatty acids in sympathotonic goats with a predominance of sympathetic nervous system activity indicated a more pronounced intensity of metabolic processes in the body. This is explained by the functional characteristics of this part of the autonomic nervous system. The sympathetic nervous system stimulates the breakdown of nutrients to provide the body with the necessary energy, which is important for maintaining homeostasis. This was reflected in the increase in the content of omega-3 fatty acids in the blood plasma, which indicated an increase in the intensity of metabolism in the body of these animals. Furthermore, goats with a predominance of sympathetic nervous system activity have a more excitable nervous system. In confirmation of this, sympathotonic goats had a higher content of docosahexaenoic acid compared to animals in other experimental groups,

which plays an essential role in ensuring the transmission of nerve impulses.

Considering the percentage of omega-6 fatty acids, it was found that the content of linoleic acid in the blood plasma of vagotonic goats was the lowest among all experimental groups ($P < 0.001$) and amounted to $20.69 \pm 0.19\%$. The level of arachidonic acid in the blood plasma of goats compared to animals of the normotonic group was 1.2 times higher in sympathotonics (7.75 ± 0.15 , $P < 0.001$) and 1.2 times lower in vagotonics (5.58 ± 0.11 , $P < 0.001$). Cis-8,11,14-eicosatrienoic acid had the highest percentage in goats with vagotonia by 2.1 times ($0.15 \pm 0.01\%$, $P < 0.001$) compared to normotonics. Indicators of unsaturated fatty acids in animals with a predominance of the parasympathetic nervous system, primarily omega-6 fatty acids in plasma lipids, were the lowest among other experimental groups (Fig. 2).

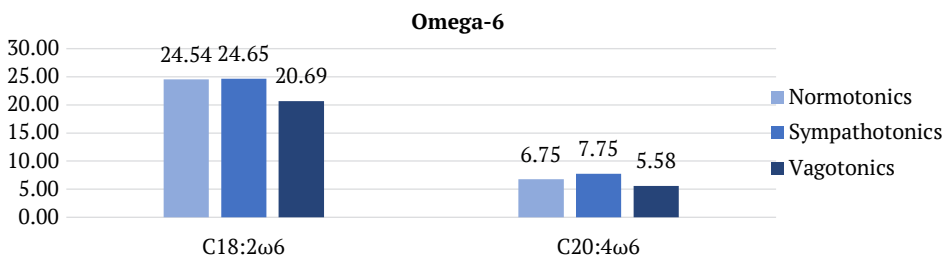


Figure 2. The content of linoleic and arachidonic acid in plasma lipids of goats of experimental groups, %

Source: developed by the author of this study

Analysing the results on the content of omega-6 fatty acids, it was found that animals with a more pronounced activity of the parasympathetic nervous system had the lowest content of these acids in the blood plasma. Considering that this part of the autonomic nervous system is responsible for processes that are opposite to the sympathetic nervous system, the predominance of vagotonia has been found to play a significant role in shaping the level of essential fatty acids in the blood plasma. Furthermore, the relative content of linoleic acid in the blood plasma of animals with the predominance of the influence of the parasympathetic nervous system on the studied processes substantially decreased compared to that of goats in other experimental groups. This omega-6 fatty acid plays a significant role in the correction of cholesterol levels – the higher its concentration, the more actively this essential fatty acid is

consumed. Since the parasympathetic nervous system affects digestion and endogenous synthesis of substances, a corresponding decrease in the content of linoleic acid, which is actively involved in them, was observed.

As for omega-9 fatty acids, cis-11-eicosenoic acid had 1.1 times higher percentage in sympathotronics ($0.51 \pm 0.01\%$) ($P < 0.05$) and 1.1 times higher percentage in vagotonics ($0.53 \pm 0.01\%$) ($P < 0.01$) compared to the experimental group of normotonics. Palmitoleic acid was the lowest in goats with a predominance of the sympathetic nervous system of $0.34 \pm 0.02\%$ ($P < 0.05$) (Fig. 3). Oleic acid, as the representative with the highest percentage concentration in the body of goats among omega-9 fatty acids, had 1.1 times lower relative content in sympathotronics ($18.02 \pm 0.20\%$, $P < 0.01$) and 0.46% lower in vagotonics ($18.46 \pm 0.10\%$, $P < 0.05$) compared to normotonics (Fig. 4).

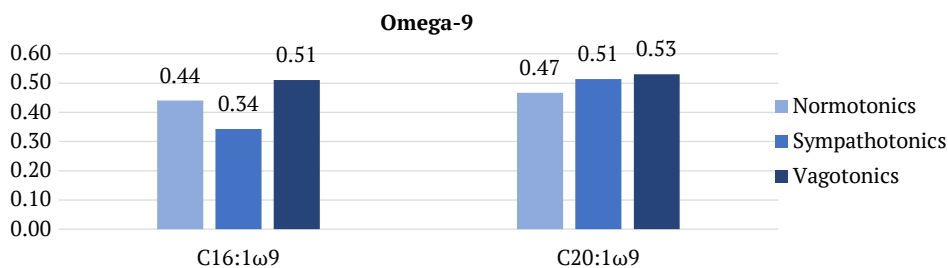


Figure 3. Content of palmitoleic and cis-9 eicosenoic acid in plasma lipids of goats of experimental groups, %

Source: developed by the author of this study

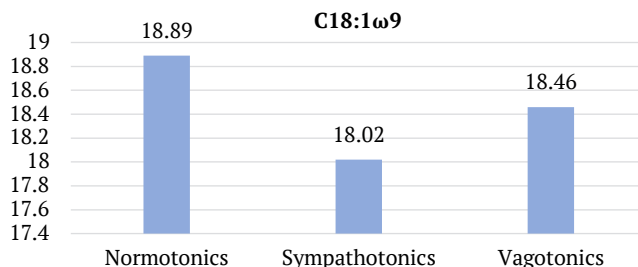


Figure 4. Oleic acid content in blood plasma lipids of goats of experimental groups, %

Source: developed by the author of this study

The content of omega-9 fatty acids in the blood plasma of sympathotonic goats was the lowest. The obtained values reflect the specific feature of the intensity of metabolic processes in these animals. Considering the impact of omega-9 fatty acids, especially oleic acid, on immunity, regeneration, and inflammation, their metabolic activity should also be accommodated. Therefore, animals with a predominance of sympathetic nervous system activity showed a more pronounced intensity of metabolism. As a result, omega-9 fatty acids are used more efficiently by the goat's body, as indicated by the findings of this study.

It is important to note the significant role of omega-3, omega-6, and omega-9 fatty acids in the body. All products obtained from the farm are used in human nutrition, both in primary and secondary form. It is an integral component of every consumer's diet, which can be implemented in various variations. Emphasis is placed on the consumption of essential fatty acids. The reason is that they cannot be endogenously synthesised in mammals. This is because they are not synthesised in the mammalian body, but come from food, mainly plant foods. The concentration of unsaturated fatty acids in the mammalian body within certain limits is crucial. These acids play a leading role in stabilising lipid metabolism. Comparing the same saturated and unsaturated fatty acids, their ratio should always be in favour of essential fatty acids. One of the first reasons is their effect on blood cholesterol levels. Due to a sufficient concentration of unsaturated fatty acids in blood plasma lipids, they are quite effective in improving the process of removing excess fat from the body. Essential fatty acids stimulate the formation of high-density lipoprotein, which transports excess cholesterol from the tissues into the bloodstream to the liver, where it is converted to bile acids and excreted in bile into the duodenum. It is also important to

highlight certain groups of unsaturated fatty acids that can substantially affect the homeostasis of lipid metabolism in mammals. These include such fatty acids as omega-3, omega-6, and omega-9 fatty acids (Czyż *et al.*, 2020; Giger-Reverdin *et al.*, 2020; Coniglio *et al.*, 2022).

According to A. Moustafa (2021), among the omega-6 fatty acids with the highest concentration is linoleic acid, which is involved in the regulation of many processes. Normal intake of this fatty acid improves the structural integrity of the skin, which also ensures its barrier function. These features are explained by the fact that this omega-6 fatty acid is part of the composition of ceramics. Linoleic acid is involved in the correction of cholesterol content in the body. It enhances the transcription of the hepatic receptor X alpha, which promotes the activation of the peroxisome proliferator. As a result, the expression of the cholesterol 7 α -hydroxylase gene, which is responsible for encoding enzymes whose main role is its conversion into bile acids, increases. As a result of the process of lipid conversion in the liver, the synthesis of high-density lipoprotein increases. This also engages proteins involved in the binding of sterol regulatory elements, which are transcription factors and play a major role in the regulation of cholesterol levels. The elements responsible for sterols activate the transcription of genes that encode the synthesis of high-density lipoprotein, cholesterol, fatty acids, and triacylglycerols. This ensures the process of lipid metabolism in the body. According to R.D. Taylor & E.H. Clayton *et al.* (2021), arachidonic acid plays a significant role in the inflammatory process. This omega-6 fatty acid makes up 25% of the fatty acids that make up the phospholipids of skeletal muscle, brain, liver, platelets, and immune cells. The deacylation and reactivation process maintains low levels of free arachidonic acid in the cell membranes, which helps prevent oxidation. The interaction

of this fatty acid with oxygen through cyclooxygenase, lipoxygenase, and cytochrome P450 pathways gives rise to the formation of mediators, commonly called eicosanoids (prostaglandins, thromboxanes, and leukotrienes). Considering this, arachidonic acid plays a major role in inflammation processes as their mediator and regulator. When omega-6 fatty acids are used as feed additives, researchers have noted their stimulating effect on metabolic processes, the functioning of the antioxidant system, and the reproductive function of the body. According to the findings of this study, goats with lower levels of omega-6 fatty acids in their blood plasma showed fewer functional features compared to animals in other groups.

J. Shunthwal *et al.* (2023) note that omega-3 fatty acids correct the course of important processes that ensure the full functioning of the animal body. Docosahexaenoic acid performs many functions, such as regulation of active transport of amino acids (choline, glycine, taurine) across cell membranes; regulation of sodium channel function and implementation of the rhodopsin response to visual stimuli. This fatty acid is the most abundant component in the central nervous system and retina. This is explained by the fact that docosahexaenoic acid supports such processes as neurotransmission, neuroplasticity, and nerve impulse transmission. This fatty acid also increases the concentration of serotonin and acetylcholine in nervous tissue and is a precursor of neuroprotectin D1. Docosahexaenoic acid has anti-inflammatory effect. It primarily affects the formation of eicosanoids. This feature is provided by the effect on the release of arachidonic acid from cell membranes, inhibition of enzymes and competition with omega-6 fatty acids for participation in enzyme-dependent metabolic processes. The feed additives described by the authors were effective in improving the balance of fatty acids. However, changes in their

concentration were not considered in the analysis of the overall course of lipid metabolism. Therewith, the findings obtained suggest that to clarify the specific features of metabolic processes, the factor of influence of the autonomic nervous system should be considered, especially in relation to the use of feed additives.

A.K. Verma *et al.* (2023) indicate that omega-9 fatty acids, specifically oleic acid, are involved in the regulation of immunity, inflammation, and tissue regeneration. For instance, when there is an injury to the skin structure, the processes of inflammation are activated. For the normal course of these stages, a large number of mediators, such as nitric oxide, is required, which is essential for rapid wound healing. This is explained by the fact that it ensures the functioning of fibroblasts, macrophages, and keratinocytes during the regenerative process. A sufficient concentration of oleic acid provides an effective anti-inflammatory effect, which occurs as a result of an increase in the supply of neutrophils to the affected tissue area, and the mediator neutrophilamine is also produced, which accelerates wound healing. The authors noted the pronounced effectiveness of using oil blends enriched with essential fatty acids. Nevertheless, slight differences in the findings obtained are noteworthy, which may be primarily due to the individual characteristics of the animals. It is quite difficult to successfully classify animals according to the criteria of the respective groups. Therefore, such studies should consider the factor of autonomic nervous system tone, which causes substantial differences in the content of omega-9 fatty acids in the blood plasma of goats.

Considering the enormous benefits of essential fatty acids, which are classified as unsaturated fatty acids, it is important to ensure that dairy products are better supplied with these substances. Such useful compounds will enrich the raw materials obtained from goat

breeding. Every day, many scientists provide increasingly more evidence of the value of these fatty acids. Due to this, people are beginning to enrich their diets with unsaturated fatty acids to prevent problems with the cardiovascular system, improve lipid metabolism in the body, etc. As a result, consumers are becoming more focused in their choice of dairy products and are forcing farmers to adjust the composition of milk and secondary raw materials. As a result of this rapid pace of development and growing demand, scientists are increasingly interested in investigating the issues that arise on goat farms. J.F. Orzuna-Orzuna *et al.* (2023) point out that one of the main challenges facing scientists is to improve animal productivity and the quality of raw materials obtained in production, while reducing the costs of solving this problem. In their research, they provide examples of the successful use of feed additives in this regard. However, according to the findings of the presented study, in conducting such studies, when forming experimental groups of animals, it is necessary to consider the individual properties of the autonomic nervous system tone, which will substantially improve the analysis and calculation of the results obtained.

R. Zhang *et al.* (2022) note that the most popular opinion among the majority is the issue of dietary adjustment. The researchers pointed out a wide range of different food additives in the form of mixed fodder from different cereals, even the addition of seaweed to the goats' diet. When analysing the researchers' recommendations on the use of feed additives to maintain the required level of essential fatty acids in animals, attention should be drawn to the significance of considering the individual characteristics of lipid metabolism depending on the tone of the autonomic nervous system.

Analysing the results of introducing an excess of essential fatty acids into the diet of goats, in most cases there was an increase in

their concentration in the body and subsequently in milk. Most often, polyunsaturated fatty acids are added to the diet of these animals. There are two schemes for enriching goats with unsaturated fatty acids. According to the findings of E. Amr *et al.* (2023), the first is to increase their content in the feed, while the second is to control biohydrogenation processes in the rumen with the subsequent accumulation of polyunsaturated fatty acids. Each of the areas has an interesting foundation for achieving the desired result. It should also be borne in mind that, despite all the positive aspects of unsaturated fatty acids, there are many undesirable consequences. The simplest problem cited by M. Afshar *et al.* (2022) lies in the deterioration of the productive state of microorganisms in the rumen. This can cause further dysfunction of the digestive system. Therefore, the use of different feed additives is not always an effective solution. The data obtained by N. Ghavipanje *et al.* (2022), R. Zhang *et al.* (2022) suggested a slight increase in unsaturated fatty acids or even no clear change. This was mainly explained by the inclusion of a small number of fatty acids in the diet to prevent their negative effects.

Considering the studies of researchers aimed at implementing the idea of increasing the content of unsaturated fatty acids in feed, the question arises as to why the body's reaction to the consumption of excess of these substances is not observed. Insufficient attention is paid to the mechanism of maintaining lipid homeostasis in the body of goats, its provision and correction. The autonomic nervous system is a good example of this, and its role as a component of a well-coordinated homeostasis regulation system reflects metabolic processes quite well. J. Imai & H. Katagiri (2022) described in detail the mechanisms of metabolic homeostasis adjustment involving the sympathetic and parasympathetic nervous systems,

especially in the correction of lipid metabolism. They gave an example of the coordinated work of these parts of the autonomic nervous system. Thus, the sympathetic nervous system not only accelerates the breakdown of nutrients in the body, but also constantly adjusts the stability of the necessary energy compounds, including glucose and lipids, to ensure the nutrition of the brain, muscles, and other tissues. In turn, the parasympathetic nervous system ensures the processes of glycogen synthesis and accumulation and the formation of adipose tissue. There was an increase in oxidative processes in animals with a predominance of the activity of the sympathetic nervous system, which contributed to an increase in the content of fatty acids in the blood due to lipid breakdown in the muscles. J. Zhang *et al.* (2023) conducted a study to determine the effect of the autonomic nervous system on the fatty acid composition of muscles in sheep. Specifically, an increase in the content of unsaturated fatty acids, especially linoleic and oleic acids, was found in animals with a predominance of sympathetic nervous system activity. This difference was explained by the fact that due to the greater activity of sympathotonia, oxidation processes in the body are accelerated, and as a result, a large number of free radicals accumulate in the blood.

According to the findings described in this study, there is a considerable difference in the content of unsaturated fatty acids in goat plasma lipids depending on the tone of the autonomic nervous system. Omega-3 fatty acids in sympathotonics have the highest concentration compared to other experimental groups of animals. Omega-6 fatty acids were characterised by low levels in vagotonics and elevated levels in goats with a predominance of sympathetic nervous system activity. Omega-9 fatty acids differed in their percentage content, but sympathotonics showed a lower concentration in

plasma lipids compared to vagotonics and normotonics. Comparing the findings obtained with the data of other researchers, J. Zhang *et al.* (2023) noted a comparable trend towards an increase in the concentration of unsaturated fatty acids in animals with a predominance of sympathetic nervous system activity. With this in mind, it is worth noting that in goats with a predominance of sympathetic nervous system activity, a considerable increase in the content of unsaturated fatty acids is observed. This feature is characterised by the increased activity of metabolic processes in the body of these animals. The sympathetic nervous system mainly regulates the breakdown of nutrients, which leads to an increase in the content of unsaturated fatty acids in blood plasma lipids. Accordingly, high levels of omega-3 and omega-6 fatty acids were noted compared to other experimental groups of animals. Goat vagotonics, which have the advantage of the influence of the parasympathetic nervous system, were characterised by lower levels of unsaturated fatty acids in plasma lipids. Animals with active vagotonia processes have an advantage in the reactions of increasing the synthesis of nutrients and their subsequent storage in reserve.

Considering the above, adjusting the fatty acid content is the key to understanding lipid metabolism, which makes it possible to counteract the growth of excess fat and maximise its positive role. When investigating a topical issue, one should consider the key mechanisms that affect the course of the synthesis and breakdown of nutrients to obtain reliable results. Such a factor as the autonomic nervous system demonstrates the distribution of animals according to the criterion of metabolic processes. This suggests the need to consider it when assessing lipid metabolism in goats. Since a clear division of animals depending on the tone of the autonomic nervous system has been established in the analysis of the content of

unsaturated fatty acids in blood plasma lipids, gradations of indicators are observed between vagotonics, sympathotonics, and normotonics.

Conclusions

It was found that the tone of the autonomic nervous system has a substantial effect on the content of unsaturated fatty acids in goat plasma lipids. This is evidenced by the high relative content of linolenic acid, cis-11-eicosanoic acid, cis-8, 11, 14-eicosatrienoic acid, arachidonic acid, docosapentaenoic acid, cis-4, 7, 10, 13, 16, 19-docosahexaenoic acid and low concentration of palmitoleic acid, oleic acid in animals with predominance of sympathetic nervous system activity compared to normotonics and vagotonics. Therewith, animals characterised by the predominance of the parasympathetic nervous system influence had a higher content of myristoleic acid, cis-11-eicosenoic acid, cis-8, 11, 14-eicosatrienoic acid and lower values of linoleic, arachidonic and cis-4, 7, 10, 13, 16, 19-docosahexaenoic acids in blood plasma lipids compared to other experimental groups of animals.

The autonomic nervous system plays a major role in regulating homeostasis in mammals. Considering this feature, it is possible to predict the course of lipid metabolism with their additional introduction into the body of goats. For

instance, if an animal has a predominance of the sympathetic nervous system over the parasympathetic nervous system, this will result in a more active breakdown of nutrients. And if one takes goats with a predominance of the parasympathetic nervous system over the sympathetic nervous system, an increase will be observed in the synthesis of metabolic products and their storage in the body's reserves. Analysing the findings of this study, a number of features of lipid metabolism in goats depending on the tone of the autonomic nervous system should be noted, which is vital to consider when adjusting the fatty acid composition of blood plasma lipids, specifically, the content of unsaturated fatty acids, in this species of animals. Consideration of individual animal characteristics should also be used in the study of homeostasis, which is crucial when assessing the nutritional and biological value of feeds in the diet for dairy goats. It is promising to investigate the effect of fatty acid supplements on the body of goats with different tone of the autonomic nervous system.

Acknowledgements

None.

Conflict of Interest

None.

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Вплив тону автономної нервової системи на вміст ненасичених жирних кислот у складі ліпідів крові в кіз

Божена Кролічевська

Кандидат технічних наук, доцент
Вроцлавський університет природничих наук
50375, вул. Ц.К. Норвіда, 31, м. Вроцлав, Польща
<https://orcid.org/0000-0002-0291-3562>

Богдан Бойчук

Аспірант
Національний університет біоресурсів і природокористування України
03041, вул. Героїв Оборони, 15, м. Київ, Україна
<https://orcid.org/0009-0000-6169-004X>

Валентин Карповський

Доктор ветеринарних наук, професор
Національний університет біоресурсів і природокористування України
03041, вул. Героїв Оборони, 15, м. Київ, Україна
<https://orcid.org/0000-0003-3858-0111>

Ігор Гришук

Аспірант
Національний університет біоресурсів і природокористування України
03041, вул. Героїв Оборони, 15, м. Київ, Україна
<https://orcid.org/0000-0003-2571-6876>

Андрій Гришук

Кандидат ветеринарних наук, доцент
Луганський національний університет імені Тараса Шевченка
36003, вул. Ковалю, 3, м. Полтава, Україна
<https://orcid.org/0000-0002-4608-337X>

Анотація. Актуальність дослідження полягає у необхідності визначення ролі автономної нервової системи в обміні ненасичених жирних кислот, насамперед, незамінних, в

організмі кіз, що поліпшить розуміння індивідуальних особливостей метаболізму в цих тварин. Використання встановлених маркерних показників крові надасть можливість здійснювати аналіз стану обміну ліпідів в організмі кіз та забезпечить підвищення ефективності методів його корекції. Метою роботи було визначення особливостей впливу автономної нервової системи на відносний вміст ненасичених жирних кислот у ліпідах плазми крові кіз. В експеримент залучали кіз породи Зааненська, з яких завдяки використанню електрокардіографічного аналізу за методикою Баєвського та залежно від тонуусу автономної нервової системи формували три дослідні групи: нормотоніки, симпатотоніки, ваготоніки. Для визначення відсоткового вмісту ненасичених жирних кислот у ліпідах плазми крові кіз використовували метод газорідинної хроматографії. Так, у кіз симпатикотоніків, які мають перевагу активності симпатичної нервової системи, відмічали високий відсотковий вміст у ліпідах плазми крові: ліноленової ($P < 0,01$), цис-4, 7, 10, 13, 16, 19-докозагексаєнної ($P < 0,05$), докозапентаєнної ($P < 0,001$) і арахідонової кислот ($P < 0,001$) порівняно з нормотоніками. У цих тварин також зафіксовано менший вміст у складі ліпідів плазми крові пальмітоолеїнової ($P < 0,05$) і олеїнової кислот ($P < 0,01$) на тлі нормотоніків. У кіз ваготоніків, в яких переважає вплив парасимпатичної нервової системи, у ліпідах плазми крові встановлено низький відносний вміст: цис-4, 7, 10, 13, 16, 19-докозагексаєнної ($P < 0,05$), лінолевої ($P < 0,001$) і арахідонової кислот ($P < 0,001$) та високий – цис-11-ейкозенової кислоти ($P < 0,01$) порівняно з нормотоніками. Встановлені особливості свідчать про істотний вплив тонуусу автономної нервової системи на кількісний перерозподіл ненасичених жирних кислот у складі ліпідів плазми крові кіз та необхідність їх врахування при аналізі стану відповідного обміну в організмі, що істотно покращить не лише результативність наукових досліджень, а й їх достовірність

Ключові слова: симпатотонія; ваготонія; нормотонія; газорідинна хроматографія; плазма



Features of topography and macroscopic structure of the digestive organs of the Yemeni chameleon (*Chamaeleo calypttratus*)

Mykola Kushch*

Doctor of Veterinary Sciences, Professor
State Biotechnological University
61002, 44 Alchevskykh Str., Kharkiv, Ukraine
<https://orcid.org/0000-0002-5280-9755>

Serhii Skachko

Graduate Student
State Biotechnological University
61002, 44 Alchevskykh Str., Kharkiv, Ukraine
<https://orcid.org/0009-0001-1229-5980>

Iryna Fesenko

PhD in Veterinary Sciences, Senior Lecturer
State Biotechnological University
61002, 44 Alchevskykh Str., Kharkiv, Ukraine
<https://orcid.org/0000-0002-6076-5545>

Olga Miroshnikova

PhD in Veterinary Sciences, Associate Professor
State Biotechnological University
61002, 44 Alchevskykh Str., Kharkiv, Ukraine
<https://orcid.org/0000-0002-8371-9023>

Olena Byrka

PhD in Veterinary Sciences, Associate Professor
State Biotechnological University
61002, 44 Alchevskykh Str., Kharkiv, Ukraine
<https://orcid.org/0000-0001-7316-2500>

Abstract. The relevance of this study is conditioned by the lack of detailed information on the structure and topography of the digestive organs of the Yemeni chameleon (*Chamaeleo calypttratus*). The purpose of this study was to find out the specific features of changes in body

Suggested Citation:

Kushch, M., Skachko, S., Fesenko I., Miroshnikova O., & Byrka, O. (2024). Features of topography and macroscopic structure of the digestive organs of the Yemeni chameleon (*Chamaeleo calypttratus*). *Ukrainian Journal of Veterinary Sciences*, 15(2), 138-156. doi: 10.31548/veterinary2.2024.138.

*Corresponding author



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weight, topography, and structure of the digestive organs of the Yemeni chameleon, to determine their morphometric parameters in animals from 1 day to 1 year of age. The research material included the tongue, oesophagus, stomach, intestines, liver, and pancreas of chameleons of different sexes of 9 age groups. The data obtained were processed by one-factor analysis of variance (ANOVA). According to the topography, macroscopic structure and surface of the mucous membrane, there are three intestines in the small intestine: duodenum, jejunum, and ileum, and two intestines in the large intestine: the colon with a diverticulum and the rectum, which passes into the cloaca. A feature of the serous membrane of the chameleon intestine is that it is coloured black by melanin. From 1 day to 1 year of age, the body weight of chameleons increased 185.9 times, the snout-vent length (SVL) increased 6.7 times, the length of the digestive tract increased 3.8 times, and the ratio of the length of the digestive tract to SVL decreased from 3.2 to 1.8 times. The most intensive increase in body weight and SVL occurred during the second and third months of life. The relative length of the small intestine in chameleons of different age groups was 65.1–81.6%, with the longest part being the jejunum. The increase in the morphometric parameters of the stomach, intestines, liver, and pancreas was asynchronous. The most pronounced changes in their relative weight were determined in animals of 2–3 months of age. The obtained materials supplement and clarify the information on the topography and structure of the digestive organs of the Yemeni chameleon, and therefore they will be useful in X-ray and ultrasound examination during veterinary manipulations

Keywords: reptiles; stomach; intestine; liver; pancreas; topography

Introduction

According to E.E.V. Crouch *et al.* (2021), various species of small reptiles are commonly traded and kept in home terrariums. As pointed out by G.S. Tang *et al.* (2020), captivity is an important measure for the conservation of endangered species and is a hot topic in conservation biology. At the same time, unbalanced nutrition, caging, lighting, substrate, temperature, and humidity can lead to stress and the development of various diseases. H.J. Sollom & H.R. Baron (2023) found that nutritional disorders in captive reptiles are still quite common despite improved knowledge of their breeding and feeding practices. As L.V. La'Toya (2023) points out, due to the specific features of compensatory mechanisms, reptiles do not immediately manifest the disease and owners seek veterinary care only when the problem is advanced and close to terminal.

According to B.J. Pinto *et al.* (2019), the reptile family *Chameleontidae* comprises over 200 species, which originate mainly from Africa and slightly extend into South Asia and Europe. They are distinguished by their zygodactyl paws, long retractable throwing tongue, which has no analogues in other vertebrates, rotating eye turrets that move independently of each other, a clinging tail, and a striking appearance, primarily due to psychedelic skin colour changes. M. Kubiak *et al.* (2020) noted that despite the popularity of chameleons as pets, these species are difficult to keep in captivity. According to A. Melero *et al.* (2023), Yemeni (veiled) chameleons (*Chamaeleo calyptratus*) and panther chameleons (*Furcifer pardalis*) are the most popular chameleons in the world, and therefore the two most commonly encountered species in veterinary practice. Furthermore, as found by

B.J. Pinto *et al.* (2019), Yemeni chameleons have attracted the attention of researchers as an important biological model for studying the role of functional morphology and evolutionary biology in the adaptation to arboreal life. However, according to K.A. Mathes *et al.* (2019), A. Meleiro *et al.* (2020), there are currently no published studies of the normal anatomy of these lizards.

According to Ardenete *et al.* (2023), involving a veterinarian early in the life of exotic companion animals can help ensure that they are properly fed from the start of their lives to support growth and create a solid foundation for a healthy transition to adulthood. There is limited or no research on the energy and nutrient requirements of exotic domestic animals of different ages, especially young animals. According to M.V. Skrypka *et al.* (2020), the morphological features of the gastrointestinal tract in reptiles have been investigated only partially and require further research. There is a need to expand the basic knowledge of the species, to conduct a thorough anatomical study of its digestive organs for further physiological, pathological, and phylogenetic research. K.A. Mathes *et al.* (2019), O.I. Tul (2021) pointed out that for the correct diagnosis and interpretation of diagnostic images of X-ray and ultrasound examination results, veterinary specialists need in-depth knowledge of the special anatomy of a particular animal species. Furthermore, according to E. Engelke *et al.* (2020), during clinical trials, the nomenclature of organs is a problem in the diagnosis of reptile diseases, which is not the only one, which can lead to misunderstandings about the affected parts of the digestive system. Therefore, it is an urgent task to develop a unified nomenclature of the internal organs of the Yemeni chameleon for practicing veterinarians, based on anatomical research methods.

The purpose of this study was to determine the specific features of topography, macro-

scopic structure of digestive organs and their age-related linear and mass parameters of the Yemeni chameleon (*Chamaeleo calypttratus*).

Materials and Methods

The study was conducted in 2022-2024 at the Department of Normal and Pathological Morphology of the State Biotechnological University (Kharkiv, Ukraine). Material for morphological studies was collected from Yemeni chameleons of 9 age groups: 1-, 7-, 14-day-old, 1-, 2-, 3-, 6-, 8-month-old, and 1-year-old chameleons (n = 5) of different sexes. All experimental studies were conducted following the European Convention for the Protection of Vertebrate Animals Used for Experimental and Scientific Purposes (1986). Animals for the research were obtained from private reptile nurseries and veterinary clinics. Some chameleons were euthanised for clinical reasons unrelated to digestive diseases. Some cadavers were obtained from a private reptile nursery located in the combat zone in the east of Ukraine, where the electricity supply was suddenly cut off, creating impossible conditions for keeping animals. Such animals died as a result of hypothermia or were slaughtered forcibly. The bodies of such animals or their organs were fixed in a 10% solution of neutral formalin.

The animals were kept in a separate room with comfortable conditions for reptiles. Adult animals were kept individually in terrariums with ventilation holes. The bottom of the terrarium was covered with paper, which was changed every week, allowing the chameleons to move around. The terrariums were equipped with bamboo branches for the animals to climb. The air temperature in the cold zone of the terrarium was maintained at 21-27°C, and in the warm zone – at 32-38°C. Along with heating and lighting provided by daylight and fluorescent lamps, the animals received ultraviolet radiation via UV lamps. The relative humidity

was 40-70%. Reptiles of 1 day to 1 year of age were fed with Jamaican field crickets (*Gryllus lo-corojo*) of different sizes, which were pretreated with calcium powder. The water was supplied continuously by drip and was fresh. The physiological condition of the animals was monitored during the keeping. Attention was paid to the behaviour, skin condition, including changes in its colour, mouth, eyes, muscle tone, limbs, cloaca, shape, and consistency of faeces.

The animals were necropsied through the abdominal wall with an incision in the median plane from the intermaxillary space to the cloacal slit to open the viscera. During the selection of internal organs, their topography, shape, size, and colour were determined. The absolute body weight of animals and organs was determined using the VLKT-500 balance and the Techniprot Waga Torsyjna-WT 250 torsion balance. The linear parameters of the organs were determined using a ruler, measuring tape, and callipers. The snout-vent length (SVL) of the animals' bodies was determined separately, as well as the length of the entire body from the top of the nose to the tip of the tail. The length of the digestive tract was determined as the length of the mouth, oesophagus, stomach, intestines, and cloaca. To describe the surface of the mucous membrane of the digestive tract, it was opened on the antimesenteric side. Subsequently, the preparations obtained in this way were photographed for documentation purposes.

The digital data were processed by one-factor analysis of variance (ANOVA). The data were analysed using the statistical analysis software Biostat LE 7.3 (AnalystSoft Inc., 2020). The difference between the values of indicators for each organ in different age groups of chameleons was determined according to the Tukey test, where the difference at $P < 0.05$ (including the Bonferroni correction) was considered significant. Numerical data in the tables are

presented as $x \pm SD$ ($x \pm$ standard deviation). Changes in body weight and length, as well as digestive system parameters of chameleons were assessed in comparison with the previous age. Considering the different lengths of different chameleon intestines in different age periods, the mean age index (MAI) was determined for their evaluation. The SVP of each intestine was determined as the arithmetic mean of 9 age-specific relative lengths of each intestine.

Results and Discussion

In the diagram of the structure of the digestive tract of the Yemeni chameleon (*Chamaeleo calyptratus*), the oropharynx, oesophagus, stomach, small and large intestine, ending in the cloaca, were identified. Thus, the digestive apparatus is comparable to that of higher vertebrates and other lizards, which is consistent with the relevant data of N. Hamdi *et al.* (2014), E. Engelke *et al.* (2020).

Three parts of the Yemeni chameleon's tongue were identified: proximal, middle, and distal. The proximal and middle parts of the chameleon's tongue occupy almost the entire oral cavity. The proximal part of the tongue is cylindrical, its dorsal surface is represented by the tongue cushion. The anterior free end of the tongue is slightly bifurcated in the horizontal plane, which creates a lingual fossa with a more forward projecting lower part. The middle part of the tongue has an elongated tube-like folded structure. Generally, the data obtained are consistent with the information of Y.A. Fouda *et al.* (2015), S. Frei *et al.* (2020) regarding the structure of the Yemeni chameleon's tongue. According to S. Frei *et al.* (2020), in the tongue of three species of chameleons – *Chamaeleo chamaeleon*, *Chamaeleo africanus*, and *Chamaeleon vulgaris* – three parts are distinguished: proximal, middle, and distal, which have some species-specific structural features. According to P.C. Wainwright *et al.* (1991), the

distal part is located among the soft tissues in the intermaxillary space.

The thoracic abdominal cavity of Yemeni chameleons is limited by the skeletal frame formed by the vertebral column and sternum,

as well as the ribs connecting them. The spinal column consists of thoracic, lumbar, and sacral vertebrae. The ribs have two segments: bony vertebral-costal and cartilaginous sternocostal (Fig. 1).

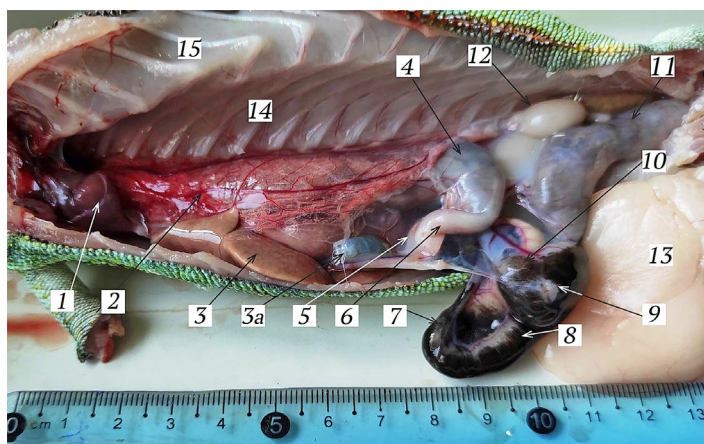


Figure 1. Organs of the thoracic-abdominal cavity

and its wall of a 1-year-old Yemeni chameleon (*Chamaeleo calyptratus*)

Notes: Left side. 1 – heart; 2 – lungs; 3 – liver; 3a – gallbladder; 4 – stomach; 5 – pancreas; 6 – duodenum; 7 – jejunum; 8 – ileum; 9 – colon; 10 – colonic diverticulum; 11 – rectum; 12 – testis; 13 – adipose body; 14 – vertebral segments of ribs; 15 – sternocostal segments of ribs

Source: developed by the author of this study

Some ribs present a third cartilaginous segment located between the vertebral-costal and sternocostal segments. The distal cartilaginous segments of several parasternal ribs merge along the midline of the abdomen and form the parasternum caudal to the sternum. Caudoventrally, it is generally delimited by a soft abdominal wall. The oesophagus enters cranially through the inlet (*apertura thoracis cranialis*), and the cloaca enters caudally through the outlet (*apertura pelvis caudalis*).

The oesophagus, a thin, short tube, is located along the axis of the body and shifts to the left before entering the stomach. There was no clear boundary between the oesophagus

and the empty stomach. When the stomach was filled with food, it was clearly distinguished by a much larger diameter than the oesophagus (Fig. 2). When empty, the stomach is cylindrical. The body of the stomach is located mainly in the right part of the thoracic cavity, dorsal to the liver and caudal vena cava, and its pyloric section is located on the left side. The pyloric part of the stomach passes without a sharp border into the small intestine, which has a much smaller diameter and is located mainly in the caudal part of the thoracic cavity. Ventral to the stomach is the spleen, which is spherical and cherry-red in colour.



Figure 2. Digestive organs of an 8-month-old Yemeni chameleon (*Chamaeleo calyptatus*)

Notes: 1 – tongue; 2 – oesophagus; 3 – stomach; 4 – duodenum; 5 – jejunum; 6 – ileum; 7 – colon; 8 – colonic diverticulum; 9 – rectum; 10 – cloaca; 11 – pancreas; 12 – liver; 13 – gallbladder; 14 – spleen

Source: developed by the author of this study

The liver of the Yemeni chameleon is located in the cranioventral part of the thoracic cavity, where it borders ventrally on its wall. It borders cranially on the apex of the heart, dorsally on the lungs, and caudally on the intestines and fatty bodies. The gallbladder is located between the lobes of the liver, has an ovoid shape, and when filled, it protrudes caudally beyond the lobes and borders the intestines and fatty bodies. The liver in the segmental plane is triangular, brownish-grey, and consists of two lobes: the right lobe, to which the gallbladder with green bile is attached, and the left lobe. The size of the right lobe of the liver has a pronounced individual variation: it is slightly or significantly larger than the left lobe, which is consistent with the data of A.A. Al-Doaiss *et al.* (2023) and A. Melero *et al.* (2023) and contradicts the data of O.I. Tul (2021), according to which the left lobe of the liver is the largest in the Yemeni chameleon. In some animals after 6 months of age, the gallbladder contents are represented by a transparent dense oval-shaped green body, which is consistent with findings of A. Gim-mel *et al.* (2017) on the detection of cholelith in the gallbladder of reptiles. The right lobe of the liver has two caudal processes: one larger in the caudal corner of the dorsal margin, the

other smaller in the middle of the dorsal margin; the left lobe has one process in the middle of the dorsal margin. A.A. Al-Doaiss *et al.* (2023) reported melanin deposits in the liver of a Yemeni chameleon. According to the findings of this study, no melanin pigmentation of the chameleon liver surface was detected.

According to S. Mans (2013), the gastrointestinal tract is the most diverse of all reptile body systems, which poses a particular challenge for veterinarians when trying to provide nutritional advice, interpret diagnostic tests, and develop treatment regimens. According to N.D. Youngblut *et al.* (2019), reptiles have a less differentiated gastrointestinal anatomy compared to mammals. Ö. Çakici & E. Akat (2013) in the snake-eyed lizard (*Ophisops elegans*), the intestine was divided only into small and large intestines, without division into separate intestines. In the Egyptian mastigure (*Uromastix aegyptiaca*), M. Zaher *et al.* (2012) also did not divide the small intestine into separate intestines. R. Diaz-Figueroa & M.A. Mitchell (2006) distinguished the small and large intestine, as well as the colon, in the reptilian intestine. According to N. Srichairat *et al.* (2018), the intestine of the Asian water monitor (*Varanus salvator*) lacks the jejunum and caecum, and

according to M.V. Skrypka *et al.* (2020), the sand lizard (*Lacerta agilis*) lacks the caecum. H.S. Lee & S. Ku (2004) identified the duodenum, small intestine, and colon in the intestine of the mountain grass lizard (*Takydromus wolteri*). There is limited information on the structure of the Yemeni chameleon's intestines. According to R. Nesas (2004), the intestinal tract of the Yemeni chameleon is short and poorly differentiated. According to N. Hamdi *et al.* (2014), the small intestine of the African chameleon (*Chamaeleo africanus*) is not divided into duodenum and ileum, and the large intestine can be divided into colon and rectum.

When dividing the small and large intestines of the Yemeni chameleon into separate intestines, this study was guided by the

detailed description of the digestive organs of the central bearded dragon (*Pogona vitticeps*), which belongs to the family *Agamidae*, provided by E. Engelke *et al.* (2020). These data were obtained based on a study of the macroscopic structure of the intestine, the relief of the mucous membrane, and its blood supply. The small intestine is located in the caudoventral part of the thoracic cavity, cranially bordering the liver and lungs, caudodorsally – the kidneys. Three intestines were identified in the small intestine of the Yemeni chameleon: duodenum, jejunum, and ileum. The duodenum is separated from the jejunum by the confluence of the pancreatic duct and bile duct. On the surface of the duodenum, there was a pancreas, which had the shape of a narrow thin strip of pale pink colour (Fig. 3).

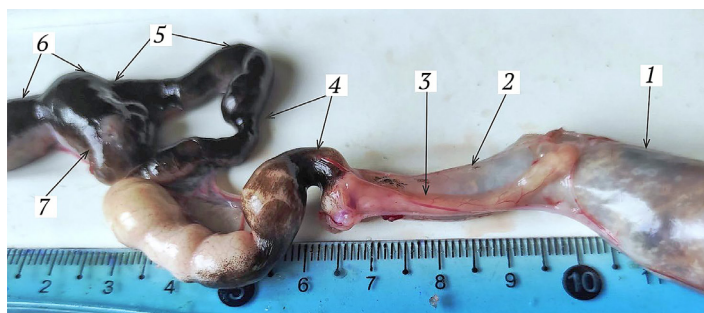


Figure 3. Digestive organs of a 1-year-old Yemeni chameleon (*Chamaeleo calypttratus*)

Notes: 1 – stomach; 2 – duodenum; 3 – pancreas; 4 – jejunum; 5 – ileum; 6 – colon; 7 – colonic diverticulum

Source: developed by the author of this study

As a result of anatomical examinations, it was found that there was no division of the pancreas into lobes. At the same time, R. Nesas (2004) points to the presence of two lobes in the pancreas of the Yemeni chameleon, although not always separate. The caecum is the longest and forms a U-shaped loop that is held in place by a long mesentery. At the end of the distal part of the loop, the caecum passes into the short ileum, which flows into the colon. The boundary between the ileum and colon is a narrow opening formed by the

ileocolic sphincter. In chameleons up to 2 months of age, the remnant of the yolk bladder was detected on the surface of the caecum in the form of a yellowish spherical formation with a diameter of 1.0-1.5 mm. As pointed out by T. Mazurkevych & S. Usenko (2023), in birds, a permanent structure is formed at the site of the remnant of the yolk duct, which connects the yolk bladder cavity with the small intestine cavity during embryonic ontogeny, – Meckel's diverticulum, which is an organ of immune defence.

Two intestines were identified in the large intestine of Yemeni chameleons: the colon and the rectum. The colon is located mainly in the middle of the whole between the right and left fatty body in the caudoventral part of the abdomen. Compared to the ileum, the colon has a much larger diameter. Its characteristic feature is the presence of a bulge – a diverticulum corresponding to the caecum. Relative to the colon, the axis of the diverticulum is directed somewhat caudally. Before passing into the rectum, the colon narrows and forms the colic isthmus. In contrast to the central bearded dragon's colon, which is spherical (ampulli-

form), as found by K.A. Mathes *et al.* (2019), the Yemeni chameleon's colon is cylindrical. The rectum has a large diameter, is located directly along the axis of the body and passes into the cloaca. Attention is drawn to the complete or partial black discolouration of the intestinal serosa, which was observed from the duodenum and extended to the rectum inclusive. There is no such information available on the structure of the intestines of both chameleons and other reptile species.

The surface structure of the mucous membrane of the digestive tract has substantial features in different parts of the tract (Fig. 4).

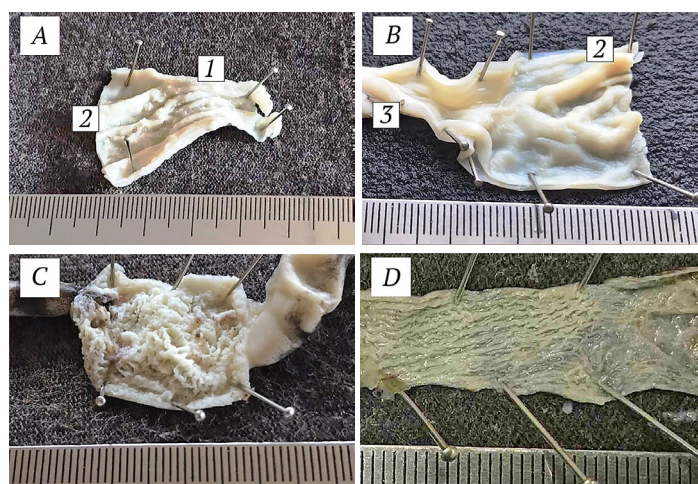


Figure 4. The surface structure of the mucous membrane of the oesophagus, stomach and small intestine of the Yemeni chameleon (*Chamaeleo calypttratus*)

Notes: A – oesophagus – stomach; B – stomach – duodenum; C – caecum; D – ileum. 1 – oesophagus; 2 – stomach; 3 – duodenum

Source: developed by the author of this study

In the oesophagus, the mucous membrane forms several longitudinal, almost parallel folds that continue to the entrance to the stomach. The gastric mucosa contains several thick folds that have different directions, which is consistent with the reports of T.S. Parsons & J.E. Cameron (1977) regarding their non-parallel direction and the presence of both wavy and straight

parts of the folds. In the duodenal mucosa, there were clearly visible thin longitudinal folds, which in the duodenum were much more numerous, thin, wavy, and had a somewhat chaotic arrangement, which is consistent with the data of T.S. Parsons & J.E. Cameron (1977). In the ileum, the number and height of the folds are smaller. The ileum and colon are separated

by the ileocecal foramen, which is based on a thick circular fold that forms the ileocolic valve. The mucous membrane of the colon and rectum contains several longitudinal folds and is separated from each other by a circular fold.

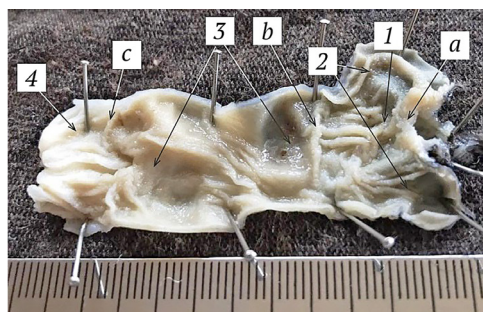


Figure 5. Surface structure of the mucous membrane of the large intestine of the Yemeni chameleon (*Chamaeleo calyptratus*)

Notes: 1 – colon; 2 – colonic diverticulum; 3 – rectum; 4 – caecum; a – ileocolic valve; b – circular fold between colon and rectum; c – circular fold between rectum and caecum

Source: developed by the author of this study

The area of the colon in front of the rectum has several distinct longitudinal folds, its

diameter decreases slightly, which corresponds to the colic isthmus. The mucous membrane of the colic diverticulum is smooth. The mucous membrane of the cloaca forms several distinct longitudinal folds. Generally, the features of the relief of the mucous membrane of the oesophagus, stomach, and individual intestines of the Yemeni chameleon are comparable to those of the central bearded dragon, which is consistent with the data of E. Engelke *et al.* (2020). Compared to 1-day-old animals, the body weight of 1-year-old Yemeni chameleons increased 185.9 times (Table 1), which corresponds to the data of A. Herrel *et al.* (2014) on the increase in body weight of this species within a year after hatching by up to two orders of magnitude. During the first month of postnatal ontogeny, the body weight of chameleons increased by 92.9%, in the second month – by 240.7%, and in the third month – by 452.3%. In the period from 3 months to 6 months of age, the increase was 197.6%, from 6 to 8 months of age – 13.6%, from 8 months to 1 year of age – 392.4%. Thus, during the observation period, the most intensive increase in chameleon body weight occurred during the second and third months of life.

Table 1. Body weight and length of Yemeni chameleons of 1 day – 1 year of age ($M \pm \text{Std}$, $n = 5$)

Age	Body weight, g	Body length, cm	SVL, cm	Digestive tract length, cm	Ratio of digestive tract length to SVL
1 day	1.4 ± 0.1^a	7.0 ± 0.5^a	3.1 ± 0.3^a	10.0 ± 1.5^{ab}	3.2 ± 0.4^c
7 days	1.8 ± 0.1^{ab}	7.4 ± 0.1^{ab}	3.3 ± 0.3^{ab}	7.5 ± 0.8^a	2.3 ± 0.3^{ab}
14 days	2.1 ± 0.1^{ab}	9.3 ± 0.1^{ab}	4.0 ± 0.2^{ab}	8.6 ± 0.7^{ab}	2.1 ± 0.1^{ab}
1 month	2.7 ± 0.3^{ab}	9.9 ± 0.2^{ab}	4.4 ± 0.4^{ab}	10.2 ± 1.9^{ab}	2.4 ± 0.1^b
2 months	6.5 ± 0.4^{ab}	13.6 ± 0.3^b	6.6 ± 0.5^b	12.8 ± 0.7^{ab}	1.9 ± 0.2^{ab}
3 months	29.4 ± 3.3^{ab}	16.0 ± 0.6^{bc}	8.1 ± 1.2^{bc}	16.3 ± 1.7^b	2.0 ± 0.1^{ab}
6 months	58.1 ± 6.3^b	26.2 ± 0.9^c	12.3 ± 1.8^c	23.7 ± 2.4^{bc}	1.9 ± 0.2^{ab}
8 months	66.0 ± 3.0^{bc}	27.3 ± 0.4^{cd}	12.9 ± 0.7^{cd}	22.6 ± 2.9^{bc}	1.8 ± 0.2^{ab}
1 year	260.2 ± 28.3^c	44.3 ± 1.0^d	20.8 ± 1.9^d	38.0 ± 6.3^c	1.8 ± 0.2^a

Notes: *different letters indicate values that differ substantially within a column based on Tukey's test ($P < 0.05$) with Bonferroni correction

Source: developed by the author of this study

The SVL of 1-year-old chameleons was 6.7 times higher than that of 1-day-olds. In

the first month of keeping these animals, it increased by 41.9%, in the second – by 50.0%,

in the third – by 62.1%, from 6 to 8 months of age – by 4.9%, and from 8 months to 1 year of age – by 61.2%. Thus, like body weight, SVL growth was most intense during the third month of life. It is during the period of the most intensive growth that chameleons are particularly sensitive to adverse environmental factors, which must be considered when breeding them. During the observation period, the general pattern of changes in the length of the digestive tract of Yemeni chameleons was its increase. The length of the digestive tract in 1-year-old animals was 3.8 times longer than in 1-day-old chameleons. At the same time, in the period from 1- to 7-day of age and from 6- to 8-months of age, a decrease was observed, which was also described by P.A. Iji *et al.* (2001), which focused on the uneven growth rate of the digestive organs. The ratio of the length of the alimentary canal to the SVL was highest up to and including 1 month of age. Subsequently, this indicator decreased and reached the lowest

values in animals of 8 months and 1 year of age.

In parallel with the increase in the weight and length of the Yemeni chameleons' bodies, the absolute weight of their stomachs and intestines also increased. Thus, the absolute weight of the stomach and intestine in 1-year-old animals was 170.5 and 226.3 times higher, respectively, compared to 1-day-old chameleons (Table 2). In the first month, these figures increased by 204.0% and 268.8%, respectively, in the second month – by 99.3% and 317.0%, in the third month – by 321.1% and 92.7%, and in the period from 3 to 6 months of age – by 95.8% and 130.0%. Compared to 6 months of age, the absolute weight of the stomach of 8-month-old chameleons was 10.8% higher, and the intestines were 23.4% lower. From the age of 8 months to 1 year, the weight of the stomach increased by 207.8%, and the weight of the intestines – by 334.1%. The relative weight of the stomach and intestines increased until 2 months of age and was lower in older animals.

Table 2. Stomach and intestine weight of Yemeni chameleons of 1 day – 1 year of age ($M \pm \text{Std}$, $n=5$)

Age	Stomach weight		Intestines weight	
	absolute, mg	relative, %	absolute, mg	relative, %
1 day	27.8 ± 4.3^a	2.0	60.8 ± 4.8^a	4.3
7 days	46.8 ± 6.2^{ab}	2.7	86.8 ± 11.6^{ab}	5.0
14 days	70.4 ± 8.4^{ab}	3.4	114.2 ± 14.2^{ab}	5.5
1 month	84.6 ± 12.6^{ab}	3.1	224.2 ± 63.0^{ab}	8.1
2 months	168.6 ± 15.5^{ab}	2.6	510.0 ± 69.0^{ab}	7.8
3 months	710.0 ± 157.2^b	2.4	$1,802.0 \pm 570.5^b$	6.0
6 months	$1,390.0 \pm 305.4^c$	2.4	$4,188.0 \pm 925.4^{bc}$	7.1
8 months	$1,540.0 \pm 152.1^{cd}$	2.7	$3,174.0 \pm 181.2^{bc}$	4.9
1 year	$4,740.0 \pm 609.9^d$	1.9	13758.0 ± 3786.4^c	5.2

Notes: different letters indicate values that differ substantially within a column based on Tukey's test ($P < 0.05$) with Bonferroni correction

Source: developed by the author of this study

Against the background of a significant increase in the absolute weight of the stomach and intestines with age, their linear indicators changed to a lesser extent. Thus, compared to

their 1-day-old age, the length and width of the stomach were 5.1 and 11.0 times longer in 1-year-old animals, respectively (Table 3), and the length of the intestine was 3.2 times greater (Table 4).

Table 3. Linear stomach parameters of Yemeni chameleons of 1 day – 1 year of age ($M \pm \text{Std}$, $n=5$)

Age	Length, mm	Width, mm
1 day	11.4 ± 0.8^a	1.4 ± 0.1^a
7 days	14.0 ± 1.0^{ab}	1.8 ± 0.1^{ab}
14 days	15.2 ± 1.6^{ab}	2.2 ± 0.2^{ab}
1 month	18.2 ± 1.1^{ab}	2.6 ± 0.1^{ab}
2 months	20.4 ± 2.1^b	5.6 ± 0.4^b
3 months	24.4 ± 1.9^{bc}	9.8 ± 0.6^c
6 months	23.8 ± 2.3^{bc}	10.6 ± 0.8^{cd}
8 months	25.4 ± 5.0^{bc}	9.4 ± 1.5^{cd}
1 year	57.6 ± 8.4^c	15.4 ± 1.8^d

Notes: different letters indicate values that differ substantially within a column based on Tukey's test ($P < 0.05$) with Bonferroni correction

Source: developed by the author of this study

Table 4. Length of intestinal sections of the Yemeni chameleon 1-day-old to 1-year-old ($M \pm \text{Std}$, $n=5$)

Age	Total length, mm	Thin section length, mm	Thick section length, mm	Relative length of the thin section, %	Ratio of intestines length to SVL
1 day	75.0 ± 13.9^{ab}	61.2 ± 12.4^{ab}	13.8 ± 1.5^a	81.6	2.4 ± 0.4^b
7 days	43.5 ± 8.5^a	28.6 ± 6.5^a	14.9 ± 2.0^{ab}	65.7	1.3 ± 0.2^{ab}
14 days	50.4 ± 3.9^{ab}	32.8 ± 3.8^{ab}	17.6 ± 2.6^{ab}	65.1	1.3 ± 0.1^{ab}
1 month	61.2 ± 4.2^{ab}	44.0 ± 4.0^{ab}	17.2 ± 1.1^{ab}	71.9	1.4 ± 0.1^{ab}
2 months	81.6 ± 10.8^{bc}	59.8 ± 9.1^b	21.8 ± 4.6^{ab}	73.3	1.2 ± 0.1^{ab}
3 months	96.8 ± 15.6^b	70.4 ± 13.8^{bc}	26.4 ± 2.2^b	72.7	1.2 ± 0.1^{ab}
6 months	156.0 ± 19.6^{cd}	121.6 ± 16.0^{cd}	34.4 ± 4.3^c	77.9	1.3 ± 0.1^{ab}
8 months	144.4 ± 13.8^c	102.6 ± 18.5^c	41.8 ± 8.3^d	71.1	1.1 ± 0.1^a
1 year	242.4 ± 35.4^d	181.0 ± 30.0^d	61.4 ± 6.1^e	74.7	1.2 ± 0.2^{ab}

Notes: different letters indicate values that differ substantially within a column based on Tukey's test ($P < 0.05$) with Bonferroni correction

Source: developed by the author of this study

According to the data obtained, in the Yemeni chameleon, the largest relative length of the small intestine was observed in animals of 1 day of age, and the smallest – in animals of 7-14 days of age. In chameleons of 1 month to 1 year of age, it was 71.1-77.9%. The high values of both absolute and relative length of the jejunum and the entire small intestine in 1-day-old animals are probably related to the significance of this intestine in digestion and absorption of nutrients from the gallbladder during the embryonic period of ontogeny. As K.D. Kohl *et al.* (2016) point out, the length of the intestine and its individual intestines is a rather labile structure that responds

quickly to the body's nutrient needs and dietary composition. According to K.D. Kohl *et al.* (2016), omnivorous Ruibal's tree iguanas (*Liolaemus ruibali*) fed plant-based food had a 17% longer small intestine than lizards fed mixed food, although no differences in small intestine weight and volume were observed. In most animals, juveniles are not simply miniature versions of adults. In fact, as identified by S.J. Gould (1977), they differ in many aspects, including morphology, physiology, locomotion, and nutritional preferences. According to K. Troyer (1984), young and adult reptiles have different needs for nutrients and specific features of the structure of their digestive organs.

Growing iguanas digest food 1.3-2.0 times faster than adults, and can reduce the time it takes to pass it.

According to the results of determining the relative length of individual intestines of chameleons of different ages and the mean

age index (MAI) of this parameter, it was found that the largest length was the caecum, which was almost half of the total length of the intestine, then in descending order of this index the intestines were located as follows: colon, duodenum, and ileum (Tables 5, 6).

Table 5. Length of the small intestine of Yemeni chameleons of 1 day – 1 year of age ($M \pm \text{Std}$, $n = 5$)

Age	Duodenum		Jejunum		Ileum	
	abs., in mm	rel., %	abs., in mm	rel., %	abs., in mm	rel., %
1 day	4.6 ± 1.4^a	6.1	53.2 ± 10.6^{bc}	70.9	3.4 ± 0.5^a	4.5
7 days	7.4 ± 1.7^{ab}	17.0	16.4 ± 4.3^a	37.7	4.8 ± 1.2^{ab}	11.0
14 days	6.6 ± 1.5^{ab}	13.1	22.0 ± 3.7^{ab}	43.7	4.2 ± 0.5^{ab}	8.3
1 month	5.6 ± 1.3^{ab}	9.1	32.0 ± 5.1^{ab}	52.3	6.4 ± 1.3^{ab}	10.5
2 months	10.6 ± 3.1^{ab}	13.0	40.2 ± 5.1^b	49.3	9.0 ± 1.5^{ab}	11.0
3 months	13.4 ± 4.4^b	13.8	45.4 ± 6.1^{bc}	46.9	11.6 ± 4.1^b	12.0
6 months	17.6 ± 4.8^{bc}	11.3	90.8 ± 8.9^d	58.1	13.2 ± 3.1^{bc}	8.6
8 months	18.8 ± 2.6^{bc}	13.0	71.4 ± 15.7^c	49.4	12.4 ± 2.3^{bc}	8.6
1 year	32.6 ± 9.3^c	13.4	121.4 ± 15.8^e	50.1	27.0 ± 7.1^c	11.1
MAI		12.2 ± 1.0		50.9 ± 3.1		9.5 ± 0.8

Notes: different letters indicate values that differ substantially within a column based on Tukey's test ($P < 0.05$) with Bonferroni correction

Source: developed by the author of this study

Table 6. Length of the large intestine and cloaca of Yemeni chameleons of 1 day – 1 year of age ($M \pm \text{Std}$, $n = 5$)

Age	Colon		Rectum		Cloaca
	abs., in mm	rel., %	abs., in mm	rel., %	abs., in mm
1 day	7.2 ± 1.3^a	9.6	6.6 ± 0.8^{ab}	8.8	3.0 ± 0.5^a
7 days	8.9 ± 1.2^{ab}	20.5	6.0 ± 0.8^a	13.8	3.0 ± 0.5^a
14 days	11.2 ± 1.8^{ab}	22.2	6.4 ± 0.8^{ab}	12.7	3.2 ± 0.5^{ab}
1 month	9.6 ± 0.9^{ab}	15.7	7.6 ± 0.7^{ab}	12.4	3.6 ± 0.5^{ab}
2 months	12.8 ± 2.7^{ab}	15.7	9.0 ± 1.9^{ab}	11.0	3.8 ± 0.8^{ab}
3 months	14.0 ± 1.8^b	14.5	12.4 ± 2.1^b	12.8	4.4 ± 0.6^{ab}
6 months	16.8 ± 2.7^{bc}	10.8	17.6 ± 2.1^{bc}	11.3	5.2 ± 0.5^b
8 months	22.2 ± 5.3^c	15.4	19.6 ± 3.5^c	13.6	5.4 ± 0.6^{bc}
1 year	33.8 ± 3.8^d	13.9	27.6 ± 3.7^d	11.4	12.2 ± 2.0^c
MAI	–	15.4 ± 1.4	–	12.0 ± 0.5	–

Notes: different letters indicate values that differ substantially within a column based on Tukey's test ($P < 0.05$) with Bonferroni correction

Source: developed by the author of this study

The data obtained suggest an uneven and asynchronous growth of the length of both individual intestines and the corresponding parts of the intestine in Yemeni chameleons,

which is a manifestation of a known biological pattern regarding the growth of this organ in other animal species, as also indicated by D. Yovchev *et al.* (2013), P.M. Gavrylin &

M.O. Nikitina (2017). For instance, during the second and third months of keeping animals, the length of the duodenum increased by 89.3% and 9.4%, respectively, the length of the jejunum – by 25.6% and 12.9%, the ileum – by 66.6% and 28.9%, the colon – by 33.3% and 9.4%, and the rectum – by 15.4% and 37.8%. Over these age periods, the length of the small intestine increased by 35.9% and 17.7%, respectively, and the length of the large intestine – by 26.7% and 21.1%.

In the first month of postnatal ontogeny in Yemeni chameleons, the absolute weight of the liver and pancreas increased by 150.0% and 209.1%, respectively, in the second month – by 192.0% and 170.6%, in the third – by 616.5% and 271.7%, in the period from 3 months to 6 months of age – by 83.7% and 51.5%, from 6 to 8 months of age – by 11.3% and 4.2%, from 8 months to 1 year of age – by 313.3% and 192.6% (Table 7).

Table 7. Morphometric parameters of the liver and pancreas of the Yemeni chameleon of 1 day – 1 year of age, $M \pm m$, $n = 5$

Age	Liver		Pancreas		length, mm
	absolute weight, g	relative weight, %	absolute weight, mg	relative weight, %	
1 day	27.0 ± 1.5^a	1.9	1.1 ± 0.3^a	0.07	5.4 ± 1.9^a
7 days	38.0 ± 6.5^{ab}	2.1	1.7 ± 0.5^{ab}	0.09	8.4 ± 1.7^{ab}
14 days	48.0 ± 3.3^{ab}	2.3	1.8 ± 0.5^{ab}	0.09	8.0 ± 1.3^{ab}
1 month	67.4 ± 16.7^{ab}	2.5	3.4 ± 1.0^{ab}	0.12	6.8 ± 1.5^{ab}
2 months	196.8 ± 34.1^{ab}	3.0	9.2 ± 2.8^{ab}	0.14	12.0 ± 3.6^{ab}
3 months	$1,410.0 \pm 409.2^{ab}$	4.7	34.2 ± 11.5^{ab}	0.12	9.6 ± 3.5^{ab}
6 months	$2,590.0 \pm 462.3^{ab}$	4.5	51.8 ± 7.3^{ab}	0.09	19.4 ± 5.1^b
8 months	$2,882.0 \pm 190.3^b$	4.4	54.0 ± 6.1^{ab}	0.07	20.4 ± 2.6^{bc}
1 year	$11,910.0 \pm 2,699.0^c$	4.6	158.0 ± 69.5^b	0.06	35.2 ± 8.1^c

Notes: different letters indicate values that differ substantially within a column based on Tukey's test ($P < 0.05$) with Bonferroni correction

Source: developed by the author of this study

The relative liver weight increased until the age of 3 months and then stayed within 4.4-4.7%. The relative weight of the pancreas increased until 2 months of age, and gradually decreased in older animals. Thus, during the observation period, the most intensive increase in both liver and pancreas weight in chameleons occurred during the third month of life, reaching a maximum value at 3 and 2 months of age, respectively.

Thus, based on the results of morphological studies, the specific features of the topography and macroscopic structure of the digestive organs of the Yemeni chameleon, as well as age-related changes in their mass and

linear parameters, were determined. The obtained findings can be useful in species morphology, for clarifying the development of digestive organs in the postnatal period of ontogeny, during diagnostic studies, for determining morphological changes in pathology, which will contribute to the development of new approaches in animal treatment.

Conclusions

Keeping reptiles as exotic pets is not an easy task for owners due to the diversity of species and lack of knowledge about their biological characteristics. Errors in feeding and housing lead to diseases of reptiles, among which one

of the most common is pathologies of the digestive system. Among reptiles, Yemeni chameleons (*Chamaeleo calypttratus*) are considered especially popular for keeping at home. The lack of information on the topography and structure of the digestive organs of these animals is a limitation for performing veterinary diagnostic procedures, especially radiographic and ultrasound. The study found that the location of the digestive organs and their macroscopic structure in the Yemeni chameleon corresponded to the general patterns of structure in lizards. The chameleon tongue has three parts: proximal, middle, and distal. The stomach has a cylindrical shape, which passes into the duodenum without a sharp border. The liver consists of two lobes: the larger right lobe, to which the gallbladder is attached, and which has two branches, and the smaller left lobe, which has one branch. The pancreas is a strip on the surface of the duodenum, not divided into lobes. According to the specific features of topography, macroscopic structure and mucosal surface structure, three intestines are distinguished in the small intestine: duodenum, jejunum, and ileum, and two intestines are distinguished in the large intestine: the colon with diverticulum and the rectum, which passes into the cloaca. The relief of the mucous membrane of the digestive tract has

features in different parts of it. In the oesophagus, the mucous membrane forms several thick longitudinal folds, and in the stomach – several thick folds with different directions. In the small intestine, the mucous membrane forms thin, numerous folds, which are wavy and more numerous in the large intestine. The mucous membrane in the colon and rectum, as well as the cloaca, has predominantly longitudinal folds, and is smooth in the diverticulum of the colon. In the period from 1 day to 1 year of age of Yemeni chameleons, morphometric parameters of the liver, pancreas, stomach, and intestines increased against the background of body weight growth, which occurred asynchronously and unevenly and most intensively at 2-3 months of age. The linear parameters of the digestive system increased to a much lesser extent compared to the weight. The period of the most intensive growth corresponded to higher relative weight of the digestive organs. In the future, it is planned to investigate the microscopic structure of the digestive organs of the Yemeni chameleon.

Acknowledgements

None.

Conflict of Interest

None.

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Особливості топографії і макроскопічної будови органів травлення єменського хамелеону (*Chamaeleo calyptratus*)

Микола Куш

Доктор ветеринарних наук, професор
Державний біотехнологічний університет
61002, вул. Алчевських, 44, м. Харків, Україна
<https://orcid.org/0000-0002-5280-9755>

Сергій Скачко

Аспірант
Державний біотехнологічний університет
61002, вул. Алчевських, 44, м. Харків, Україна
<https://orcid.org/0009-0001-1229-5980>

Ірина Фесенко

Кандидат ветеринарних наук, старший викладач
Державний біотехнологічний університет
61002, вул. Алчевських, 44, м. Харків, Україна
<https://orcid.org/0000-0002-6076-5545>

Ольга Мірошнікова

Кандидат ветеринарних наук, доцент
Державний біотехнологічний університет
61002, вул. Алчевських, 44, м. Харків, Україна
<https://orcid.org/0000-0002-8371-9023>

Олена Бирка

Кандидат ветеринарних наук, доцент
Державний біотехнологічний університет
61002, вул. Алчевських, 44, м. Харків, Україна
<https://orcid.org/0000-0001-7316-2500>

Анотація. Актуальність дослідження зумовлена відсутністю детальної інформації стосовно будови і топографії органів травлення єменського хамелеону (*Chamaeleo calyptratus*). Мета роботи полягала у з'ясуванні особливостей змін маси тіла, топографії і будови органів травлення єменського хамелеону, визначенні їх морфометричних показників у тварин від 1-добового до 1-річного віку. Матеріалом досліджень слугували язик, стравохід, шлунок, кишечник, печінка і підшлункова залоза хамелеонів різної статі 9 вікових груп. Отримані дані обробляли методом однофакторного дисперсійного аналізу (ANOVA). За особливостями топографії, макроскопічної будови і поверхні слизової оболонки в тонкому відділі кишечника виділено три кишки: дванадцятпалу, порожню і клубову, а в товстому – дві кишки: ободову з дивертикулом і пряму, що переходить у клоаку. Особливістю серозної

оболонки кишечника хамелеонів є забарвлення меланіном в чорний колір. З 1-добового до 1-річного віку маса тіла хамелеонів збільшувалась в 185,9 раза, довжина тіла від верхівки носа до анального отвору (SVL) – в 6,7 раза, довжина травного каналу – у 3,8 раза, відношення довжини травного каналу до SVL зменшувалось з 3,2 до 1,8 раза. Найінтенсивніше зростання маси тіла і SVL відбувалось впродовж другого і третього місяців життя тварин. Відносна довжина тонкого відділу кишечника в хамелеонів різних вікових груп становила 65,1-81,6 %, у складі якого найдовшою була порожня кишка. Збільшення морфометричних показників шлунка, кишечника, печінки і підшлункової залози відбувалось асинхронно. Найвиразніші зміни показників їх відносної маси визначали в тварин 2-3-місячного віку. Отримані матеріали доповнюють і уточнюють інформацію стосовно топографії і особливостей будови органів травлення єменського хамелеону, тому будуть корисними в рентгенографічному і ультразвуковому дослідженні під час проведення ветеринарних маніпуляцій

Ключові слова: рептилії; шлунок; кишечник; печінка; підшлункова залоза; топографія



The role of zoophilic flies *Hermetia illucens* (Diptera: Stratiomyidae) in the spread of myiasis in mammals

Serhii Melnychuk*

Doctor of Biological Sciences, Professor
National Academy of Agrarian Sciences of Ukraine
01010, 9 Mikhail Omelyanovich-Pavlenko Str., Kyiv, Ukraine
<https://orcid.org/0009-0006-6583-3128>

Andrew Dubovyi

PhD in Medical Sciences
The University of Auckland
1023, 85 Park Road, Auckland, New Zealand
<https://orcid.org/0000-0003-1978-9163>

Nataliia Soroka

Doctor of Veterinary Sciences, Professor
National University of Life and Environmental Sciences of Ukraine
03041, 15 Heroiv Oborony Str., Kyiv, Ukraine
<https://orcid.org/0000-0003-4659-6666>

Serhii Honcharov

Doctor of Veterinary Sciences, Associated Professor
National University of Life and Environmental Sciences of Ukraine
03041, 15 Heroiv Oborony Str., Kyiv, Ukraine
<https://orcid.org/0000-0001-7464-6689>

Abstract. The relevance of investigating the distribution, biology, and role of zoophilic flies *Hermetia illucens* of the family *Stratiomyidae* in the occurrence of myiasis in mammals lies in the ability of these flies to parasitise animals, which poses a threat to their health and life. The purpose of this review was to analyse scientific research on the possible spread of *Hermetia illucens* flies as a potential pathogen of parasitic diseases in Ukraine. The study employed the methods of analysis, comparison, and synthesis of scientific sources. It was found that the natural habitat of these flies is the countries of South and North America with tropical and subtropical climates. However,

Suggested Citation:

Melnichuk, S., Dubovyi, A., Soroka, N., & Honcharov, S. (2024). The role of zoophilic flies *Hermetia illucens* (Diptera: Stratiomyidae) in the spread of myiasis in mammals. *Ukrainian Journal of Veterinary Sciences*, 15(2), 157-169. doi: 10.31548/veterinary2.2024.157.

*Corresponding author



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these flies are also found in European countries, namely: Portugal, Spain, Malta, France, Albania, Croatia, Slovenia, and Switzerland. To intensify biotechnology in the agricultural sector, the larvae of *Hermetia illucens* flies are used as an ecological utiliser of organic agricultural residues. It is believed that the main reason for the emergence of *Hermetia illucens* flies in Europe is their importation into seaports and subsequent introduction into coastal areas. Considering the above, it is possible that *Hermetia illucens* flies may also appear in Ukraine (in the southwestern regions). Cases of larval parasitism of these flies in mammals have been established. They were mostly detected as parasites in the gastrointestinal tract. Their ability to cause typical enteric myiasis has been confirmed. Larval parasitism is non-specific: diarrhoea (faeces with blood and mucus), flatulence, vomiting, loss of consciousness, etc. The larvae can also parasitise under the skin, forming boils. A frequent cause of human infestation is the consumption of unwashed, overripe fruit and vegetables on which these flies lay their eggs. The results of the review can be used to develop methods for controlling the spread and reproduction of *Hermetia illucens* flies, as a species whose larvae are capable of parasitising mammals

Keywords: entomoses; saprophytic insects; larvae; facultative parasite; hermetiosis

Introduction

Even though flies are generally considered pests and spread infectious and parasitic diseases, they are important in providing alternative sources of protein. Specifically, the *Hermetia illucens* Linnaeus, 1758 or the *black soldier fly* has significant potential for large-scale production of various biotechnological products through the processing of organic waste. According to M. Zamri *et al.* (2023), this is because insects are a source of complete protein, vitamins, and amino acids. At the same time, growing insects on an industrial scale has many significant benefits, such as reducing greenhouse gas emissions and providing more sustainable economic opportunities. Researchers have noted the use of *H. illucens* pupae for feeding cattle, pigs, and fish, and the fat from these larvae is used to produce biodiesel (Kannan *et al.*, 2023). The use of an alternative protein obtained from *H. illucens* flies has a positive effect on the growth rate of fish raised in aquaculture, as well as on the biochemical parameters of blood serum and the microbiological composition of the gastrointestinal tract of fish (Busti *et al.*, 2024). In the current conditions of livestock intensification,

this species of fly is of great interest because it is an excellent “utiliser” of organic waste, a producer of high-quality protein for animal feeding and chitin and chitosan for the pharmaceutical industry. Components derived from *H. illucens* also demonstrated a prominent level of growth inhibition for all mycelia studied – *Alternaria solani*, *Botrytis cinerea*, *Fusarium oxysporum*, *Pythium capsici*, *Sclerotinia sclerotiorum* (Kaczor *et al.*, 2023). According to A. Makarynska *et al.* (2022), the current popularity of using *H. illucens* flies at different stages of development in modern biotechnology is considered to be safer for the environment and can make a substantial contribution to climate protection.

H. illucens belongs to the order *Diptera*, family *Stratiomyidae*, suborder *Brachycera* *Orthorrhapha*, infrasuborder *Stratiomyomorpha*. This species of fly is common in North and South America. *H. illucens* flies have been reported to be widespread worldwide, specifically in countries and regions with tropical, subtropical, and temperate climates (Kaczor *et al.*, 2023). At the same time, A. Amir *et al.* (2020) reported the possibility of larval stages of *H. illucens* to

parasitise the gastrointestinal tract of animals and humans and cause enteric myiasis or hermatitis. Furthermore, it is noted that the incidence of mammalian enteric myiasis caused by *H. illucens* larvae is widespread in regions with a low level of socio-economic development. This is primarily due to the feeding of spoiled and rotten fruit or vegetables to animals, on which flies lay their eggs beforehand.

The purpose of this review was to analyse the special scientific literature on the role of zoophilic flies *H. illucens* in the spread of myiasis in animals and humans and their biology, as well as the risks of these flies in Ukraine as a potential facultative parasite.

The study was based on the methods of analysis, comparison, and systematisation of scientific literature and the findings of other studies. The scientific literature was analysed regarding the role of flies of the genus *Hermetia*, namely *Hermetia illucens*, in the spread of myiasis in mammals. This analysis provided information on known cases of myiasis caused by zoophilic flies, including their geographical distribution, mammalian host species, characteristics of the fly parasitoid stages, and impact on host health. Next, the results of different studies on the role of *Hermetia illucens* in the spread of myiasis were compared to identify similarities and differences in the data. This approach helped to identify general patterns and specific features in the impact of zoophilic flies on mammalian health and welfare. The main part of the study was to systematise the collected information to create a comprehensive understanding of the role of *Hermetia illucens* in the spread of myiasis in mammals. Notably, the current studies cover exclusively the issues related to the use of zoophilic flies *H. illucens* for biotechnological purposes. This includes domestic research by Ukrainian scientists on the use of flies for feeding vertebrates and aquatic life. At the same time, a considerable number of reports on *H. illucens*

parasitism in animals and humans date back to the second half of the 20th century. This fact is apparently related not so much to the decrease in cases of *H. illucens* parasitisation of flies as to the lack of methods for diagnosing hermatitis in mammals, which makes the topic extremely relevant.

Features of biology and morphology of zoophilic flies of the family *Stratiomyidae*

According to R.A. Rozkosný (1983), *Hermetia illucens* usually reproduces in underutilised compost from street latrines and poultry droppings, rotting vegetables and fruits, animal carcasses and military waste, and other decomposing organic residues. Fly larvae are more likely to be found in humid environments than in dry ones. Adults have mouthparts that are not adapted to feed on animal and human blood. That is why *H. illucens* flies are not vectors of blood-borne pathogens.

A. Martínez-Sánchez *et al.* (2011) reported the possibility of egg laying and larval development of *H. illucens* in human cadavers. Thus, in the corpse of an elderly man in the late stages of autolysis found in Northern Spain, larvae of *H. illucens* were found in large numbers. In addition, larvae and pupae of *H. illucens* flies were found in human corpses subjected to decay in Italy (Turchetto *et al.*, 2001). At the same time, in the southern, central, and western United States and Hawaii, *H. illucens* fly larvae are saprophytes of superficially buried human remains (Lord *et al.*, 1994).

L. Newton *et al.* (2005) noted that *H. illucens* flies undergo five stages of the developmental cycle: egg, larva, pre-pupa, pupa, and adult (female and male). Depending on the temperature and other environmental conditions, pupation lasts from 9 days to 5 months. That is why in their natural habitats, the first flight of these flies can be recorded as early as April, but most of them reach sexual maturity only in late

summer. The larvae look for sheltered and dry places to pupate. Specimens, females and males, emerging from the pupa immediately copulate in the air during flight. Soon after, the fertilised females begin to lay eggs on the edges of the accumulation of decaying organic residues. The eggs take 4 days to 3 weeks to mature, after which the larvae hatch. The latter undergo several stages of development before pupating.

In the first stages of development, as proven by D.C. Hall & R.R. Gerhardt (2002), the larvae are dull, slightly whitish in colour after hatching. They have a clearly visible protruding head with a well-developed chewing apparatus. The larvae undergo three moulting stages and take approximately 30 days to complete their development. During this period, the larvae have an extraordinary appetite and are voracious. However, as adults, flies do not need nutrients and use fats stored during the larval stage. The pupal stage is formed inside a specific skin formation – the puparium.

Thus, according to N.E. Woodley (2001), the puparium is characterised by circular hair growth on the last segment and may be somewhat retracted. The puparium of zoophilic flies of the genus *Hermetiinae* is morphologically characterised by an elongated body consisting of eight segments. There are no spikes on the surface of the puparium. B.M. Drees & J. Jackman (1999) pointed out that some larvae of zoophilic flies of the family *Stratiomyidae* are aquatic or semi-aquatic. During their development, they feed on algae, decomposing organic matter and aquatic organisms. The larvae can also use animal and human faeces, rotting fruit and vegetables, or under the bark of decaying wood as a nutrient substrate.

Sexually mature individuals are black to metallic blue with green-purple or black-yellow patterns (Bondari & Sheppard, 1981). Adult flies have a body length of 15 to 20 mm. Their wings are quite dense, smoky black. In

the resting state, flies have their wings pressed against the dorsal part of their bodies (Tomblerlin *et al.*, 2002; Üstüner *et al.*, 2003) (Fig. 1).



Figure 1. *H. illucens* fly

Source: M. de Groot & P. Veenliet (2011)

According to F. Oliveira *et al.* (2015), the head of adult *H. illucens* flies is small and narrower than the body. Their eyes are faceted, divided in both sexes. The antennae have a long terminal segment with an elongated flagellum. The antennae are twice as long as the head. The abdomen of flies has 8 segments, which are formed by rectangular plates. Furthermore, their entire abdomen is covered with numerous small bristles that look longer from the caudal angle. These flies have pronounced sexual dimorphism: the female is larger than the male. Males have a short genital apparatus represented by two pairs of lateral lobes. The reproductive apparatus of females is represented by a genital furca. The anal opening in adults looks like a slit in the abdominal cavity of their body.

The zoophilic flies *H. illucens* are characterised by seasonality. Thus, in France, adult flight begins in late April and lasts until 15 November (Alvarez, 2012; Cocquempot & Martinez, 2017). In Spain, the mating season can begin on 2 March and last until 19 November, as in Albania (Üstüner *et al.*, 2003). Thus, zoophilic flies *H. illucens* are characterised by a saprophytic lifestyle, which lies in using various organic residues of animal or plant origin as energy

material to ensure the full growth and development. That is why the predominant area of distribution of these flies is associated with places of accumulation of faeces, rotting vegetables and fruits, etc. The life cycle of *H. illucens* flies is characterised by seasonality and a full development cycle consisting of five stages.

Distribution of the zoophilous fly *Hermetia illucens* and results of its introduction

M. Hejda *et al.* (2019) pointed out that in recent decades, invasive species of plants and animals, including insects, have become extremely widespread. This spread of biologically invasive species is recognised as a serious international problem. Certain introduced insect species do not have a considerable negative impact on the habitat. However, some of their species can multiply rapidly and spread to new territories and have a significant negative impact on ecosystems, animal and human health, and can be dangerous pests of crops. Invasive organisms are a crucial factor in transforming natural ecosystems, reducing biodiversity and causing significant economic damage.

As reported by T. Üstüner *et al.* (2003), the current issue of zoophilic flies is their ever-increasing adaptation to unfavourable environmental conditions, and, accordingly, the growth of their range of settlement. Under natural conditions, the population of zoophilic flies is in constant interaction with populations of various host animals, which are integral components of the biocenosis. From an environmental standpoint, the use of these flies at different stages of development is an alternative source of animal protein. Therewith, flies consume fewer resources and provide reserves of high-quality nutrients (Józefiak *et al.*, 2016). At the same time, the efficiency with which *H. illucens* larvae can process various organic substrates is the highest among other fly species. Specifically, the

introduction of *H. illucens* larvae into poultry manure in poultry houses leads to a 50% reduction in its accumulation and 94-100% inhibition of house fly (*Musca domestica* L.) development (Díclaro & Kaufman, 2015). Furthermore, *H. illucens* larvae can change the microflora of poultry manure, actually reducing the number of harmful bacteria, specifically *Escherichia coli* and *Salmonella* (Erickson *et al.*, 2004).

In Panama, *H. illucens* flies have been reported to have a considerably negative impact on banana crops. Female flies lay their eggs on ripe yellow fruits. The hatched larvae quickly penetrate the fruit, leaving dark spots on the fruit. Such bananas lose their consumer appeal and spoil quickly (Stephens, 1975).

According to D.P. Furman *et al.* (1959), *H. illucens* flies are not considered to be vectors of pathogenic pathogens or to be harmful to humans. However, there are many species of zoophilic flies known to transmit anthrax, tuberculosis, brucellosis, paratyphoid, coccidiosis, and other contagious diseases in animals and humans (Lord *et al.*, 1994).

According to the findings of the study by E. McCallan (1974) and T. Üstüner *et al.* (2003), it was proved that the natural distribution area of *H. illucens* flies is the countries of North and South America within the range from 40° north latitude to 40° south latitude. In South America, this species of fly is distributed from Chile, Argentina, Uruguay to the United States. The northernmost occurrences of *H. illucens* flies in North America are known in the states of California, Kansas, Iowa, Indiana, Pennsylvania, and along the coasts of Massachusetts and New Hampshire. On the African continent, zoophilic *H. illucens* flies are found from South Africa and Madagascar to Mali; from the Eastern region of Nepal, India, and Sri Lanka to Thailand, Malaysia, and Vietnam, as well as to the Japanese islands of Ryukyu, Taiwan, Indonesia (Java and Sulawesi), and the Philippines. The Australian

and oceanic portions of the range of these flies include the Indonesian island of Irian Jaya, Papua New Guinea, Western Australia, Queensland, New Zealand, Benin Islands, Northern Mariana Islands, Guam, Belau, Marshall and Solomon Islands, Micronesia, New Caledonia, Vanatu, French Polynesia, and Hawaii (Woodley, 2001).

However, according to J.K. Tomberlin *et al.* (2002), the lack of resistance of *H. illucens* flies to low temperatures excluded their distribution in Northern Europe. It was found that flies require an optimum ambient temperature of 27°C and 60% humidity to lay eggs (Sprangers *et al.*, 2017), as well as appropriate light intensity and space (Heussler *et al.*, 2018). However, according to T. Üstüner *et al.* (2003), *H. illucens* flies are characterised by eurythermia, a species that can tolerate significant changes in air temperature.

In the Western Palaearctic region, especially in Europe, the presence of *Hermetia illucens* flies has been recorded in several countries. Specifically, they have been found in Malta, Albania, Croatia, Portugal, and Spain, as mentioned by M. Carles-Tolra (2001). According to other researchers, this species was also found in France (Chevin, 1986; Richoux, 2009; Cocquempot & Martinez, 2017). Italy was mentioned in the studies by G. Troiano & E. Toscano (1997), Switzerland – by W. Sauter (1989) and Slovenia – by M. de Groot & P. Veenvliet (2011). This fly species has also been recorded in Turkey and Syria (Üstüner *et al.*, 2003) and in Asian countries, namely in China (Wang *et al.*, 2023).

At the same time, cases of *H. illucens* detection in Europe were mainly recorded along the seacoast and near seaports. However, cases of inland penetration of this species have been confirmed in Switzerland, where *H. illucens* was found 190 km from the nearest seacoast. Subsequently, such populations successfully reproduced in compost heaps and migrated to more northern areas. The Alps Mountain range is a

natural barrier to flies and their larvae, which cause hermetiosis, to the north of Europe. For this reason, some scientists believed that the spread of *H. illucens* flies and their larvae occurs during transport through Mediterranean ports (Üstüner *et al.*, 2003). Specifically, the occurrence of *H. illucens* flies in Slovenia is the result of their importation as a means of biological control of house flies (*Musca domestica* L.) and the use of larval feed for animal feeding. At the same time, M. de Groot & P. Veenvliet (2011) pointed out that the full impact of these flies on the country's natural biodiversity has been understudied.

According to E. Molchanova *et al.* (2021), it was proved that scientific research on the possibility of using *H. illucens* fly larvae in the processing of plant waste has already been conducted in Ukraine. Thus, the natural distribution area of zoophilic *H. illucens* flies is the territories of South and part of North America, as well as a range of countries with tropical and subtropical climates. Despite claims that *H. illucens* flies cannot be spread in Northern Europe due to their demanding temperature conditions, there are reports of their presence in a large part of Europe, including Switzerland. Due to the tendency for zoophilic flies to spread through maritime trade routes, one can expect *H. illucens* to enter Ukraine through the seaports of the Black and Azov Seas, given the growth trends in global trade. At the same time, conducting experimental research with *H. illucens* flies and their larvae may pose a risk of their introduction into the natural environment of Ukraine.

Cases of parasitism of zoophilic flies *Hermetia illucens* in mammals

Even though *H. illucens* flies are classical saprophytic organisms that feed mainly on organic residues, there are described cases of their larvae parasitising animals and humans.

At the same time, other flies of the *Stratiomyidae* family, specifically *Chrysomya bezziana* Villeuve, 1914, which are common in countries with tropical and subtropical climates, are known to cause intestinal myiasis. Cases of larval parasitism in humans were first identified and described by researchers in the 1950s. Furthermore, it was reported that *C. bezziana* larvae can cause genitourinary, intestinal, and skin myiasis in humans (Oothuman & Jeffery, 1984). Later, H.L. Lee (1985) described a case of larvae infestation of the human oral cavity.

Cases of hermetiosis were recorded in animals. A case of myiasis was reported in cattle of the Simmental breed, one year old, from the village of Sucila, Yucatan (Mexico). Chronic tympanic disorder was observed in the animal for 13 days. Despite treatment, the animal died. During the pathological autopsy of the animal's corpse, 20 *H. illucens* larvae at the fourth stage of development were found in the rumen. Some larvae were fixed on the surface of the mucous membrane, while others were freely located in the scar cavity. Inflammation and haemorrhage were observed in the scar mucosa (Manrique-Saide et al., 1999). A case of enteric myiasis caused by *H. illucens* fly larvae was reported in a dog from Buenos Aires, Argentina. The larvae were found in the dog's faeces, which contained blood and mucus. According to P.R. Mulieri et al. (2019), a dog became infected with the eggs of the hermetiosis pathogen by eating previously mown lawn grass that was being composted.

H.E. Meleney & P.D. Harwood (1935) noted that one of the first cases of fly larvae infestation of the boy's digestive tract was described in the USA. He was diagnosed with all the clinical signs of hermetiotic disease. The disease was characterised by symptoms of stomach and rectum damage with loss of consciousness. Furthermore, a case of classical enteric myiasis, namely hermetiotic disease, in a seven-year-old girl from Kuala Kanchagar,

Malaysia, is described. The girl had recurrent acute manifestations of gastrointestinal tract lesions, accompanied by frequent vomiting. During the period of infection, the girl vomited 1-2 times a month. Along with vomit, fly larvae were isolated 3-4 times during the disease, which were identified as *H. illucens* by parasitological examination. Apart from vomiting, no other symptoms were observed. According to H.L. Lee et al. (1995), the larvae developed in the cavity of the child's digestive tract. Another case of *H. illucens* larvae parasitism in several human cadavers in Malaysia was reported. It was noted that the fly larvae corresponded to a developmental stage that could have been reached only during the lifetime of humans (Sinniah et al., 1994).

O. Calderón-Arguedas et al. (2005) reported a case of enteric myiasis caused by *H. illucens* larvae in a 71-year-old woman from a geriatric hospital in Costa Rica. The patient's faecal examination revealed eggs of the nematode *Ascaris lumbricoides*. The woman was treated with Mebendazole. After treatment, the woman was observed to pass *H. illucens* larvae in her faeces. A case of *H. illucens* larvae parasitising the gastrointestinal tract of a 26-year-old woman from Cuba was also reported. The woman was admitted to hospital with symptoms of abdominal pain. Herbal infusions such as southernwood (*Artemisa abrotanum* L.), mint (*Mentha nemorosa* Wild) and chamomile (*Matricaria chamomilla* L.) were used for its treatment. However, during the treatment, *H. illucens* larvae were released along with the faeces. A gastroscopic examination revealed chronic inflammation of the gastric mucosa with erosions of various sizes (González & Oliva, 2009). A case of enteric myiasis was also reported in a 26-year-old man from Sarawak, Malaysia. The patient had diarrhoea with blood impurities for one week. During defecation, fly larvae were found in the faeces. Using molecular genetic methods,

it was established that the larvae belong to the species *H. illucens*. According to A. Amir *et al.* (2020), the symptoms of intestinal myiasis caused by the black soldier fly are non-specific and could range from asymptomatic to acute abdominal pain, diarrhoea, vomiting, and loss of consciousness. At the same time, there is currently no clinically proven treatment for people with hermetiosis.

According to R.F. Harwood & M.T. James (1970), human infection with larvae occurs when consuming spoilt and rotten fruit and vegetables contaminated with *H. illucens* eggs. The researchers believed that fly larvae are quite resistant to the acidic environment of the human digestive tract, and their spiracles can close tightly, which is an additional adaptive mechanism to adverse conditions. At the same time, A.I. Adler & F.P. Brancato (1995) reported that hermetiosis can also manifest itself in the form of skin myiasis. A case of a furuncle in a woman from Seattle, Washington, who had travelled to East Africa, is described. As a result of surgical intervention, a larva of *H. illucens* was isolated from the furuncle cavity. The larvae of other flies, namely *Chrysomya bezziana* of the *Stratiomyiadae* family, also cause ear myiasis. N. W. Ahmad *et al.* (2009). reported a case of larvae in the ears of a newborn child from Malaysia.

Thus, even though *H. illucens* flies are predominantly saprophytic, numerous cases of animal and human infestation by their larvae have been described. This suggests that these flies also can lead a parasitic lifestyle when their larvae penetrate the body. When the larvae enter the mammalian body, they cause mechanical and toxic effects, which is clinically manifested by the development of non-specific symptoms.

Conclusions

The natural distribution area of zoophilic flies *H. illucens* is countries with tropical and subtropical climates. However, due to the intensification of maritime trade routes,

H. illucens flies have been successfully introduced into the coastal areas of a range of European countries, and have been recorded in some Northern European countries, including Switzerland. Considering the detection of *H. illucens* flies in Slovakia, one should expect reports of their occurrence in Ukraine in the near future. The presence of Ukraine's seaports is likely to create an additional factor contributing to the spread of these flies. Therefore, considering the above, it is likely that the southwestern regions of Ukraine will be the place of potential fly spread. These flies are typical obligate, and according to some reports, conditionally facultative saprophytes that use organic residues for life and development. However, under certain conditions, they can parasitise mammals. The vast majority of cases associated with parasitism by *H. illucens* larvae were characterised by lesions of the digestive tract in animals and humans with the development of enteric myiasis. Most cases of hermetiosis are reported in humans. The disease was accompanied by non-specific symptoms of gastrointestinal disorders. Only a few cases in animals have been described in the available literature, which probably indicates not so much the rarity of *H. illucens* larvae parasitism as insufficient diagnostic studies in case of possible invasion. Human infestations are associated with the consumption of unwashed, spoiled, and rotten fruit and vegetables on which *H. illucens* flies lay their eggs. In most of these cases, it is the violation of hygiene requirements that causes human infection. Therefore, outside the natural habitat of parasitic fly species, there is a constant need for additional research on their migration routes, biocenological relationships, phylogeny, and ontogeny, biological features, including reproduction, possible negative impact on humans and animals, economic losses, as well as the development of measures to control their reproduction and spread. The use of *H. illucens* flies in the biological

conversion of organic waste, animal feeding, etc., requires a thorough risk assessment for the areas where they are introduced, as well as the development of technologies that will consider environmental safety elements.

Further research is planned to develop preventive measures to avoid infection of animals and humans with *H. illucens* zoophilic fly larvae,

as well as clinical trials for the further treatment of hermetiotoxic disease.

Acknowledgements

None.

Conflict of Interest

None.

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Роль зоофільних мух *Hermetia illucens* (Diptera: Stratiomyidae) в поширенні міазів у ссавців

Сергій Мельничук

Доктор біологічних наук, професор
Національна академія аграрних наук України
01010, вул. Михайла Омеляновича-Павленка, 9, м. Київ, Україна
<https://orcid.org/0009-0006-6583-3128>

Андрій Дубовий

Кандидат медичних наук
Університет Окленда
1023, 85 Park Road, м. Окленд, Нова Зеландія
<https://orcid.org/0000-0003-1978-9163>

Наталія Сорока

Доктор ветеринарних наук, професор
Національний університет біоресурсів і природокористування України
03041, вул. Героїв Оборони, 15, м. Київ, Україна
<https://orcid.org/0000-0001-7464-6689>

Сергій Гончаров

Доктор ветеринарних наук, доцент
Національний університет біоресурсів і природокористування України
03041, вул. Героїв Оборони, 15, м. Київ, Україна
<https://orcid.org/0000-0001-7464-6689>

Анотація. Актуальність вивчення питання поширення, особливостей біології та ролі зоофільних мух *Hermetia illucens* родини *Stratiomyidae* у виникненні міазів у ссавців полягає

у здатності зазначених мух паразитувати в організмі тварин, що виявляє загрозу для їх здоров'я та життя. *Мета огляду* – здійснити аналіз наукових досліджень щодо можливого поширення мух *Hermetia illucens*, як потенційного збудника паразитарних захворювань, на території України. Під час дослідження було використано методи аналізу, порівняння та узагальнення наукових джерел. Встановлено, що природним ареалом поширення цих мух є країни Південної та Північної Америки з тропічним і субтропічним кліматом. Проте, цих мух виявляють і в країнах Європи, зокрема: Португалії, Іспанії, Мальті, Франції, Албанії, Хорватії, Словенії та у Швейцарії. Для інтенсифікації біотехнологій аграрного сектору, личинок мух *Hermetia illucens* використовують в якості екологічного утилізатора органічних решток сільськогосподарського виробництва. Вважається, що основною причиною появи мух *Hermetia illucens* в Європі є їх завезення у морські порти та подальша інтродукція на території прибережних зон. Враховуючи означене, можлива поява мух *Hermetia illucens* і в Україні (у південно-західних регіонах). Встановлено випадки паразитування личинок цих мух в організмі ссавців. Переважно їх виявляли за паразитування у шлунково-кишковому каналі. Підтверджено їх здатність викликати типові ентеральні міази. Паразитування личинок перебігає неспецифічно: діарея (фекалії з домішками крові і слизу), метеоризм, блювання, втрата свідомості тощо. Личинки також можуть паразитувати і під шкірою, формуючи фурункули. Частою причиною інвазування людини є споживання немитих перезрілих овочів та фруктів, на яких ці мухи відкладають яйця. Результати огляду можуть бути застосовані для розробки методів контролю за поширенням та розмноженням мух *Hermetia illucens*, як виду, личинки якого здатні паразитувати в організмі ссавців

Ключові слова: ентомози; сапрофітні комахи; личинки; факультативний паразит; герметіоз

УКРАЇНСЬКИЙ ЧАСОПИС ВЕТЕРИНАРНИХ НАУК

Науковий журнал

Том 15, № 2. 2024

Заснований у 2010 р. Виходить чотири рази на рік

Оригінал-макет видання виготовлено
у відділі науково-технічної інформації
Національного університету біоресурсів і природокористування України

Відповідальний редактор:

Г. Івченко

Редагування англomовних текстів:

С. Воровський, К. Касьянов

Комп'ютерна верстка:

О. Глінченко

Підписано до друку 29 травня 2024 р. Формат 70*100/16

Умов. друк. арк. 13,8

Наклад 100 прим.

Адреса видавництва:

Національний університет біоресурсів і природокористування України

03041, вул. Героїв Оборони, 15, м. Київ, Україна

Тел.: 380953452722

E-mail: info@veterinaryscience.com.ua

<https://veterinaryscience.com.ua/uk>

UKRAINIAN JOURNAL OF VETERINARY SCIENCES

Scientific journal

Volume 15, No. 2. 2024

Founded in 2010. Published four times per year

The original layout of the publication is made in the Department of Scientific
and Technical Information of National University of Life
and Environmental Sciences of Ukraine

Managing Editor:

H. Ivchenko

Editing English-language texts:

S. Vorovsky, K. Kasianov

Desktop publishing:

O. Glinchenko

Signed for print of May 29, 2024. Format 70*100/16
Conventional printed pages 13.8
Circulation 100 copies

Editors office address:

National University of Life and Environmental Sciences of Ukraine
03041, 15 Heroiv Oborony Str., Kyiv, Ukraine
Tel.: 380953452722
E-mail: info@veterinaryscience.com.ua
<https://veterinaryscience.com.ua/en>