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Effectiveness of nutrient media for the recovery of lyophilisates of fastidious microorganisms: evidence from *Streptococcus spp.*

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Abstract. To restore the biological and morphological properties of fastidious bacteria during lyophilization, one of which is a type of streptococcus – bacteria of the genus *Streptococcus*, it is necessary to use expensive specialized nutrient media that are limited in availability for laboratories. In this regard, the purpose of this study was to find the most effective methods of preservation and recovery of fastidious microorganisms using the example of *Streptococcus spp.* The study was performed by a bacteriological method. The isolates of *Streptococcus spp.*, which had the property of alpha- or beta-haemolysis, were selected for the study. The microorganisms were collected as a result of bacteriological research of pathological and biological materials from 20 animals (10 dogs and 10 cats). Microorganisms were determined and counted using Vitek 2 compact, easySpiral, and Scan 500 systems. As a result, the effectiveness of using various combinations of nutrient media for cryopreservation, freeze-drying, and further revitalization of cultures was proven. It was found that the most effective medium for lyophilization is meat-peptone broth with the addition of 5% bovine blood serum, diluted 1:1 with Faibich's medium, and for recovery after lyophilization – meat-peptone broth with the addition of 5% blood serum cattle and 5% glucose. With this combination, the concentration of viable cells corresponded to the limits of 1.7×10^6-2.4×10^6 CFU/cm^3. The use of other combinations of nutrient media for the revitalization of *Streptococcus* bacteria showed lower efficiency, which corresponded to the concentration of viable cells within 1.2×10^5-2.1×10^6 CFU/cm^3. The obtained results increase the efficiency of the method of lyophilization of demanding cultures due to combination of non-selective nutrient media and components available in laboratory practice

Keywords: bacteria, cryopreservation, lyophilization, culture medium, *Streptococcus*

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Introduction

Bacteria of the genus *Streptococcus spp*. can cause various diseases, ranging from mild skin infections to necrotizing fasciitis, which can threaten the health of not only animals, but also humans (Belstrom *et al*., 2021). This genus consists of both commensal species that colonize the skin and nose, as well as pathogenic and opportunistic strains that can be isolated for various diseases: upper respiratory tract, pneumonia, meningitis, sepsis, otitis, ophthalmitis, hepatitis, endometritis, and aerosacculitis (Weiser *et al*., 2018; Kim *et al*., 2018; Chang *et al*., 2022).

Some types of streptococci are one of the main colonizers and normal flora of the oral cavity – *Streptococcus mitis* and *S. oralis*, which protect it from inflammatory processes by producing hydrogen peroxide, neutralizing acids, and secreting antimicrobial compounds, competing with opportunistic and pathogenic microorganisms. The competitive properties of these bacteria open the possibility for the use of cultures such as *S. mitis* and *S. oralis* in the bacteriotherapy of diseases in combination with antibacterial preparations (Thurnheer & Belibasakis, 2018; Kim *et al*., 2018; Park *et al*., 2020).

Streptococci are less common in the environment than staphylococci (Belstrom *et al*., 2021). They are not resistant to the influence of physical environmental factors, die quickly, and are demanding to the cultivation conditions. Therewith, if cultivation and storage conditions are not observed, bacteria lose their virulence, which reduces their scientific value for researchers and practical use (Nasran *et al*., 2020).

Success in the commercialization of strains of *Streptococcus spp*. as potential valuable agents for several types of research largely depends on their preservation technologies. Appropriate methods can maintain high cell viability not only during the conservation process, but, most importantly, during the long stay of these strains in collections (Halim *et al*., 2017). Because these bacteria have vulnerable characteristics, freeze-drying under vacuum is often considered one of the best methods for preserving cells (Bircher *et al*., 2018; Bodzen *et al*., 2020).

Freeze drying, or lyophilization, is a dehydration process that stabilizes bacteria to maintain their long-term viability until use. This process is often used for production or valuable collection strains of bacteria (Bodzen *et al*., 2021) and is the most efficient way of long-term storage of microbial strains in collections. The quality of restoration of biological and morphological properties of lyophilized demanding bacteria directly depends on the culture media (Boey *et al*., 2022). The technological stress that occurs during the freeze-drying process can cause cell damage and, in some cases, their death (Bodzen *et al*., 2021). Dehydration of cells caused by osmotic stress due to the addition of protective solutes and freezing itself is a source of mechanical and structural stress on cells and cellular components. During severe dehydration, the plasma membrane can be permeabilized, which is often the cause of cell death. Proceeding from this, the choice of an effective protective environment to reduce the effects of the above factors is a crucial step for effective lyophilization. For this purpose, colloidal substances are used, which reduce the temperature point at which water crystallization occurs. This, in turn, extends the time for cooling the cells and creates conditions for minimizing the negative effect of ice crystals on these microbial cells. Another major step in sublimation drying is rehydration, which precedes the use of bacteria and affects both their survival and functionality (Oslan *et al*., 2017; Bodzen *et al*., 2021).

In the study of Ukrainian scientists Ushkalov *et al*. (2021), who conducted studies on the lyophilization of some pathogenic bacteria, also used the method of different combinations of nutrient media to revitalize cultures, namely a gram-positive coccal culture of *S. aureus*. Therewith, the highest productivity in reconstituted crops was observed upon the use of heart-brain broth.

Haindl *et al*. (2022) investigated the effect of *in vitro* freeze-drying on beneficial faecal microbiota for potential use in the treatment of gastrointestinal diseases. They found that freeze-drying affects cell number, saturation, diversity, and microbial composition, but these effects can be reversible and disappear upon re-cultivation.

Nicco *et al*. (2020) investigated the effect of cryopreservation on the survival of gastric microflora using various temperature conditions from -20°C to -196°C. High survival of microorganisms was noted in the temperature range from -70°C to -120°C.

Meneghel *et al*. (2017) described the effect of several types of sugars on cell membrane stability during freezing of cell cultures. The use of complex sugars resulted in greater destruction of microbial walls than the use of monosaccharides. Similar experimental study with comparable results was performed by Vekeman *et al*. (2013), which also involved the use of distinct types of sugars for cryopreservation of nitrite-oxidizing bacteria.

The purpose of this study was to find the most effective and practical methods for long-term storage and subsequent recovery of alpha- and beta-haemolytic streptococci isolated from dogs and cats.

Materials and Methods

The study was conducted in 2020-2021 in the Ukrainian Laboratory of Quality and Safety of Products of the agro-industrial complex of the National University of Life and Environmental Sciences of Ukraine and the bacteriological laboratory of the Kyiv Regional Clinical Hospital.

The isolates of *Streptococcus spp*., which had the property of alpha- or beta-haemolysis, were selected for the study. The microorganisms were collected as a result of bacteriological research of pathological and biological materials from 20 animal patients (10 dogs and 10 cats) of the "Multivet" Veterinary Clinic (Kyiv region).

Isolates of *Streptococcus spp*. was collected as follows: using a sterile cotton swab pre-moistened with a 0.2% TWEEN 20 solution (Merck, Germany). Swabs with the selected material were immersed in a test tube with Amies transport medium (BioTesMed, Ukraine) and transported to the bacteriological laboratory. The tampon material was seeded on selective and differential diagnostic nutrient media: 5% blood agar (GRASO, Poland) and meat-peptone broth (HiMedia, India) with the addition of 5% glucose. The inoculated cups were incubated in a thermostat at 37°C for 24 hours. For further research, colonies were selected, which, according to the results of bacterioscopy, were formed by gram-positive coccal microflora. The final identification of microorganisms was carried out using a bacteriological analyser Vitek 2 compact (bioMérieux, France).

Identified cultures of *Streptococcus spp*. underwent the process of cryopreservation and subsequent sublimation (lyophilization) as follows: as a cryoprotectant, Faibich's protective medium was used (gelatine – 1%; sucrose – 10%, distilled water – 89%) in a ratio of 1:1 with broth containing 24-hour cultures of *Streptococcus spp*. Therewith, meat-peptone broth (HiMedia, India) was used in two variations: pure meat-peptone broth and meat-peptone broth with the addition of 5% bovine blood serum to 95% of the broth volume. Each test culture was packed with 1.0 cm^3 in bottles with a volume of 5 cm^3. Before starting lyophilization, three vials were selected from each experimental batch to establish the typicality, uniformity, and concentration of bacterial mass in the suspension (CFU/cm^3). The experimental cultures were cryopreserved in a Forma 700 Series freezer (Thermo Scientific, USA) at -70°C. An LP–3 machine (TelStar, Spain) was used to ensure the lyophilization process. Lyophilization was performed at a deep vacuum of 0.17 mbar and a condenser temperature at 45-50°C.

After 6 months, pre-lyophilized cultures were revitalized using different combinations of nutrient media. For this, the following combinations of nutrient media were tested: pure meat-peptone broth; meat-peptone broth with the addition of up to 95% of the broth volume of 5% bovine blood serum; meat-peptone broth with the addition of 5% glucose to 95% of the broth volume; and meat-peptone broth with the addition of 5% bovine serum and 5% glucose to 90% of the broth volume. After 24 hours, the broth cultures were tested for typicality, uniformity, and concentration of viable microbial cells (CFU/cm^3) by seeding on blood agar using the easySpiral system (Interscience, France) and counting on Scan 500 (Interscience, France).

Statistical analysis was performed in a Microsoft Excel 2016 spreadsheet. The Online Epitools Epidemiological Calculator was used to estimate the 95% confidence interval (CI) (Epitools Epidemiological Calculators..., 2018). $P<0.05$ was considered a statistically significant result.

Results and Discussion

During the study of pathological and biological materials from 20 patient animals (10 dogs and 10 cats), 5 isolates of *Streptococcus spp*. were isolated, which had alpha- (Fig. 1) or beta-haemolysis (Fig. 2) and were used in further research (Table 1).

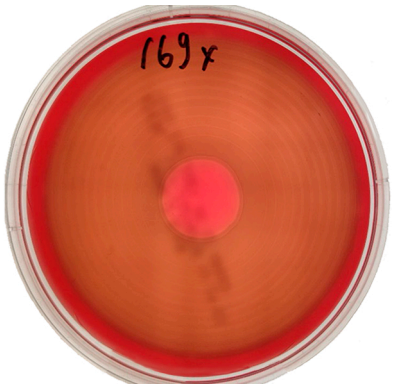

Figure 1. Alpha-haemolysis of *S.equinius* isolate on blood agar

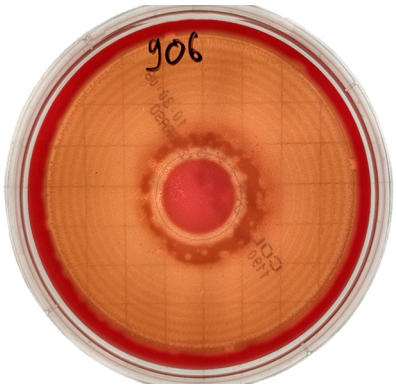

Figure 2. Beta-haemolysis of *S.agalactiae* B isolate on blood agar

Table 1. Place of isolation, type of haemolysis, and concentration of viable cells of isolates of *Streptococcus spp*. under study

Indicator	*S. agalactiae* A	*S. agalactiae* B	*S. anginosus*	*S. equinius*	*S. pseudoporcinus*
Place of isolation	Wound (dog)	Wound (cat)	Mouth (dog)	Oral cavity (cat)	Nasal cavity (dog)
Type of haemolysis	*β*	*β*	*α*	*α*	*α*
Concentration of viable cells, CFU/cm^3	1.9×10^8	4.9×10^8	1.6×10^9	1.7×10^9	1.8×10^9

Therewith, the isolates that were taken from the pathological material (from the wound) had a positive reaction to alpha-glucosidase, Ala-Phe-Pro arylamidase, phosphatase and leucine arylamidase according to the results of biochemical analysis on the Vitek 2 compact system and were identified as *S. agalactiae* A, and *S. agalactiae* B. An isolate taken from the pharynx of a healthy animal was positive for D-amygdalin, arginine dihydrolase 1, Ala-Phe-Pro arylamidase, phosphatase and leucine arylamidase and was identified by the system as *S. anginosus*. An isolate taken from the oral cavity of a healthy animal showed a positive reaction to D-amygdalin, cyclodextrin, leucinarylamidase, and L-pyrrolidonyl-arylamidase and was identified by the system as *S. equinius*. An isolate taken from the nasal cavity of a healthy animal showed a positive reaction to Ala-Phe-Pro arylamidase and leucine arylamidase and was identified by the system as *S. pseudoporcinus* (Table 2).

Table 2. Biological properties of the *Streptococcus spp*. isolates under study

Completed test	*S. agalactiae A*	*S. agalactiae B*	*S. anginosus*	*S. equinius*	*S. pseudoporcinus*
D-amygdalin	–	–	+	+	–
Phosphatidylinositol-phospholipase C	–	–	–	–	–
D-xylose	–	–	–	–	–
Arginine dihydrolase 1	–	–	+	–	–
beta-Galactosidase	–	–	–	–	–
alpha-Glucosidase	+	+	–	–	–
Ala-Phe-Pro arylamidase	+	+	+	–	+
Cyclodestrin	–	–	–	+	–
L-aspartatarylamidase	–	–	–	–	–
beta-Galactopyranosidase	–	–	–	–	–
alpha-Mannosidase	–	–	–	–	–
Phosphatase	+	+	+	–	–
Leucinarylamidase	+	+	+	+	+
L-Prolinarylamidase	–	–	–	–	–
beta-Glucuronidase	–	–	–	–	–
alpha-Galactosidase	–	–	–	–	–
L-Pyrrolidonyl-arylamidase	–	–	–	+	–

Note: *"+" – 95-100% positive reaction; "–" – 0-5% positive reaction*

The isolates under study were cryopreserved and then lyophilized using two different media. For 6 months, freeze-dried crops were stored in the refrigerator at +2–+8°C.

Table 3 presents the results of revitalization of cultures after 6 months using pure meat-peptone broth before lyophilization without the addition of bovine blood serum. Therewith, the survival rate of isolates compared to the concentration of viable cells before lyophilization using pure meat-peptone broth was 0.16-1.07%, meat-peptone broth with the addition of 5% glucose – 0.46-1.68%, meat-peptone broth with the addition of 5% bovine blood serum – 0.33-3.89%, meat-peptone broth with the addition of 5% bovine blood serum and 5% glucose – 1.05-5.26%.

Table 3. The concentration of viable cells of *Streptococcus spp.*, restored with different nutrient media by lyophilization with pure meat-peptone broth

Revitalization medium	Concentration of viable cells after lyophilization, CFU/cm^3				
	S. agalactiae A	*S. agalactiae B*	*S. anginosus*	*S. equinius*	*S. pseudoporcinus*
Meat-peptone broth	1.7×10^5	2.4×10^5	7.9×10^5	5.8×10^5	3.0×10^5
Meat-peptone broth + 5% bovine blood serum	7.4×10^5	1.2×10^6	1.7×10^6	1.6×10^6	2.0×10^6
Meat-peptone broth + 5% glucose	3.2×10^5	4.9×10^5	8.1×10^5	7.9×10^5	6.1×10^5
Meat-peptone broth + 5% glucose + 5% bovine blood serum	1.0×10^6	1.6×10^6	2.1×10^6	1.8×10^6	2.1×10^6

Table 4 presents the results of revitalization using meat-peptone broth with the addition of 5% bovine blood serum before lyophilization. Therewith, the survival rate of isolates compared with the concentration of viable cells before lyophilization using pure meat-peptone broth was 0.25-1.04%, meat-peptone broth with the addition of 5% glucose – 0.36-2.47%, meat-peptone broth with the addition of 5% bovine blood serum – 1.05-4.68%, meat-peptone broth with the addition of 5% bovine blood serum and 5% glucose – 1.17-8.94%.

Table 4. The concentration of viable cells of *Streptococcus spp.* revitalized with different nutrient media by lyophilization with meat-peptone broth with the addition of 5% bovine blood serum

Revitalization medium	Concentration of viable cultures after lyophilization, CFU/cm³				
	S. agalactiae A	*S. agalactiae B*	*S. anginosus*	*S. equinius*	*S. pseudoporcinus*
Meat-peptone broth	1.8×10^5	5.1×10^5	1.0×10^6	7.7×10^5	4.5×10^5
Meat-peptone broth + + 5% bovine blood serum	8.9×10^5	1.3×10^6	1.9×10^6	1.8×10^6	2.1×10^6
Meat-peptone broth + + 5% glucose	4.7×10^5	$5.5*10^5$	$1.2*10^6$	$1.1*10^6$	$6.6*10^5$
Meat-peptone broth + + 5% glucose + 5% bovine blood serum	1.7×10^6	1.9×10^6	2.3×10^6	2.0×10^6	2.4×10^6

Cultures of *Streptococcus spp*. required special cultivation conditions and complex nutrient media containing blood or its serum. In turn, they were used for cryopreservation, lyophilization, and subsequent revitalization of these cultures.

Thus, in case of the cryopreservation procedure, the most effective was the preliminary cultivation of cultures of *Streptococcus spp*. with the addition of 5% bovine blood serum to the broth. All cultures that were lyophilized with the introduction of this nutrient medium showed a higher survival rate (0.25-8.94%) compared to the use of pure meat-peptone broth (0.16-5.26%) for revitalization in all combinations of nutrient media (Table 4).

Using various combinations of nutrient media to revitalize streptococci after lyophilization, the highest survival rates were obtained when using meat-peptone broth with the addition of 5% glucose and 5% bovine blood serum (1.17-8.94%) when lyophilizing meat-peptone broth with the addition of 5% bovine blood serum. Other combinations of nutrient media showed lower efficiency, in which the recovery rate was lower (0.25-5.26%). This phenomenon can be explained by the tenderness and fastidiousness of streptococcal cultures, in which the use of additives that enrich non-selective media with additional nutrients provides a higher probability of these cultures escaping sublimation. Therewith, the isolates under study stayed viable during the use of all combinations of nutrient media.

The results obtained coincide with the study of Ukrainian scientists Ushkalov *et al*. (2021), which determined the effectiveness of various nutrient media in revitalizing cultures after lyophilization. The composition and nutritional value of the media substantially affected the efficiency of revitalization of lyophilized cultures. At the same time, media with higher nutritional value were marked by a large amount of CFU during revitalization after lyophilization. It should also be noted that this study uses the most common nutrient media and components in laboratory practice, which increases the accessibility of this method.

The results of the study revealed some discrepancies with the study of Haindl *et al*. (2022). In this study, cultures of *Streptococcus spp*. almost did not alter their cultural and morphological properties after lyophilization and maintained their scientific and practical qualities even from the first reinoculation. This is primarily because unlike the research methodology of R. Haindl *et al*. (2022), in this study single isolates of alpha- and beta-haemolytic streptococci were used for lyophilization, rather than a pool of cultures.

The studies of Nasran *et al*. (2020) and Bodzen *et al*. (2021) indicated that an increase in the colloid of the liquid that is part of the cell walls of cultures contributes to a better preservation of the viability of these cultures after the lyophilization process, which is also followed in the presented study – the addition of glucose to nutrient media increased the resistance of microbial cells to lyophilization drying and ensured a higher percentage of their survival compared to combinations of environments in which glucose was not applied.

It is also important to note the studies of Oslan *et al*. (2017) and Bircher *et al*. (2018), in which the results of the study of different nutrient media for cryopreservation are presented. As in this study, it was shown that the use of a medium with added sugars contributed to better preservation of microorganisms during their freezing, which is explained by the lower destructive effect of water crystals on microbial cells.

Therewith, the studies of Boey *et al*. (2022) and Vekeman *et al*. (2013), which used various types of complex and simple sugars, found the destructive effect of complex sugars on bacterial cell walls. The established fact is explained by a violation of osmotic regulation of bacteria during binding to cell wall molecules. At the same time, the use of simple sugars, on the contrary, increased the resistance of bacteria to cryopreservation. This coincides with the results of the present study, where simple sugars were used for cryopreservation. With this combination of glucose-containing nutrient media, bacterial resistance, and survival after the lyophilization process were higher than using a combination of glucose-free nutrient media.

In the study of scientists Nicco *et al*. (2020) indicate the optimal temperatures for cryopreservation of bacteria using various freezing methods. At the same time, the presently described method in case of applying a temperature of -70°C was characterized by obtaining the same limits on culture survival that these scientists described in their research.

Conclusions

The process of cryopreservation and subsequent lyophilization causes a considerable load on the microbial cell. In case of *Streptococcus spp*. cells, the greatest efficiency of cryopreservation and lyophilization, in which the survival of cultures was the highest, was obtained upon combination

of meat-peptone broth with the addition of 5% bovine blood serum with Faibich's medium 1:1 for cryopreservation and meat-peptone broth with the addition of 5% bovine blood serum and 5% glucose for revitalization of cultures after lyophilization.

The use of pure meat-peptone broth with Faibich's medium 1:1 compared to the use of meat-peptone broth with the addition of 5% bovine serum before lyophilization shows a lower percentage of survival of isolates of *Streptococcus spp*. using all combinations of revitalization media.

The use of other combinations of nutrient media for revitalization shows less efficiency but keeps a sufficient number of colony-forming units for further practical application of revitalized cultures.

To improve the efficiency of lyophilization and make it more accessible to revitalize cultures that are demanding on the composition of nutrient media and cultivation conditions, it is possible to use cheaper available nutrient media and components that are widely used in laboratory practice.

In the future, it is planned to continue research on identifying effective and practical methods for long-term storage and subsequent revitalization of alpha- and beta-haemolytic streptococci.

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Ефективність поживних середовищ для відновлення ліофілізатів вибагливих мікроорганізмів на прикладі *Streptococcus spp.*

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Анотація. Для відновлення біологічних і морфологічних властивостей вибагливих бактерій при ліофілізації, одними з яких є вид стрептококи – бактерії роду *Streptococcus*, необхідно застосовувати дороговартісні спеціалізовані поживні середовища, які мають обмежену доступність для лабораторій. У зв'язку з цим, метою роботи було визначення найефективніших методів збереження та відновлення вибагливих мікроорганізмів на прикладі *Streptococcus spp*. Дослідження виконане бактеріологічним методом. Для дослідження відбиралися ізоляти *Streptococcus spp.*, які мали властивість до альфа- або бета-гемолізу. Збір мікроорганізмів проводили у результаті бактеріологічного дослідження патологічного і біологічного матеріалів від 20 тварин (10 собак і 10 котів). Визначення та підрахунок мікроорганізмів здійснено за допомогою систем Vitek 2 compact, easySpiral і Scan 500. У результаті проведених досліджень доведено ефективність застосування різних комбінацій поживних середовищ для кріоконсервації, ліофілізації та подальшого оживлення культур. Встановлено, що найефективнішим середовищем для ліофілізації є м'ясо-пептонний бульйон з додаванням 5 % сироватки крові великої рогатої худоби, розведений 1 : 1 з середовищем Файбіча, а для відновлення після ліофілізації – м'ясо-пептонний бульйон з додаванням 5 % сироватки крові великої рогатої худоби і 5 % глюкози. За такої комбінації концентрація життєздатних клітин відповідала межам від 1.7×10^6 до 2.4×10^6 КУО/см^3. Використання інших комбінацій поживних середовищ для оживлення бактерій роду *Streptococcus* виявляло меншу ефективність, що відповідало концентрації життєздатних клітин у діпазоні від 1.2×10^5 до 2.1×10^6 КУО/см^3. Отримані результати підвищують ефективність методу ліофілізації вибагливих культур завдяки використанню комбінації доступних у лабораторній практиці неселективних поживних середовищ і компонентів

Ключові слова: бактерія, кріоконсервація, ліофілізація, культуральне середовище, стрептокок

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Hormonal regulation of the concentration of glucose and its derivatives in the blood of dairy cows during the transit period

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Abstract. In dairy cows, metabolic disorders are mainly found in the last weeks of the dry period and the first weeks after calving (transit period). Therefore, the purpose of this study was to investigate the hormonal regulation of the concentration of glucose and its derivatives in the blood of dairy cows during the transit period. The study was conducted on cows of 2-4 lactations, with a capacity of 7.8-8.2 thousand kg of milk for previous lactation. Blood for research was taken from cows 7-10 days before calving and Days 2-4, Days 10-14, and Days 30-40 after calving. The concentration of glucose in blood plasma was determined by the glucose oxidase method, pyruvate – by the modified Umbright method, lactate – by reaction with paraoxydyphenyl, and hormone content – by enzyme-linked immunosorbent assay. It was established that high-performance dairy cows during the transit period experience substantial changes in carbohydrate metabolism and the functional state of organs and systems, which are aimed at ensuring high productivity. Thus, hypoglycaemia is found in cows within two weeks after calving. At the same time, with a decrease in the concentration of glucose in the blood plasma of cows, the content of pyruvate and lactate increases, as well as the lactate/pyruvate ratio, which indicates an increase in gluconeogenesis. Negative energy balance and increased gluconeogenesis lead to a decrease in the synthesis of insulin and insulin-like growth factor. Compared to the final dry period, on Days 2-4 of lactation, the concentration of leptin in the blood plasma of cows decreased threefold and stayed at a low level until Day 40 of lactation. Plasma cortisol levels were highest on Days 2-4 and 10-14 of lactation. Intensive cortisol synthesis during the period of energy deficiency increases gluconeogenesis, which is possible due to lipolysis and proteolysis. In the first days after calving, the content of thyroxine and triiodothyronine in the blood plasma of cows decreased. Inhibition of thyroid hormone production is a consequence of the physiological regulatory features of this period. Thus, in highly productive cows during the transit period, attention should be paid to maintaining vital body functions and their well-coordinated endocrine regulation, which will ensure a physiologically balanced metabolic rate, successful calving, high milk productivity, and animal health

Keywords: dairy cattle breeding, carbohydrate metabolism, lactate, pyruvate, hormones

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Introduction

In recent decades, cow productivity has considerably increased in countries with highly developed dairy cattle breeding (Barkema *et al*., 2015). Today in Ukraine, dairy productivity has exceeded 6,500 kg on average, and in the best farms it has reached 10,000 kg or more per cow per year (State Statistics..., 2021). A high level of milk productivity requires a sufficient amount of nutrients and vital biologically active substances for the body. Therefore, in highly productive dairy cows in the last weeks before and the first after calving, there is an increase in metabolism (Stivanin *et al*., 2021). In the specialized literature, this period is called transition (Knob *et al*., 2021). For highly productive dairy cows, it is the crucial stage that determines the state of their health and the calves born from them, and in general – the level of productivity and profitability of the farm.

In the final stage of pregnancy, the main expenditure of nutrients is spent on fetal growth, placental development, and breast preparation for lactation. After calving, cows lose body weight from the main body depots due to considerable energy needs and insufficient supply of energy deriving from feed. Due to the inability to ensure that the body receives enough energy to cover the body's needs and milk production, highly productive dairy cows have a negative energy balance at the beginning of lactation (Soonberg *et al*., 2021). In the first days after calving, the lack of energy is first compensated by the breakdown of glycogen in the liver and muscles. However, glycogenolysis quickly fades, since glycogen reserves are enough only for the first two to three days, and with a considerable energy deficiency – even less (Soonberg *et al*., 2021; Cabezas-Garcia *et al*., 2021). Therefore, to cover the energy deficit, a highly productive cow uses reserve fats that are deposited during the mobilisation and dry periods.

A slight energy deficit that occurs in cows after childbirth is considered a physiological phenomenon. The milk productivity of cows is maximum in the first two months after calving, but currently the assimilation of basic nutrients from the feed is insufficient to provide them to the body. First of all, an energy deficiency occurs, which causes rapid weight loss and increases the risk of metabolic diseases in animals (Vossebeld *et al*., 2022). Excessive and prolonged intake of lipids causes insufficient oxidation in the tricarboxylic acid cycle and activation of ketogenesis (Mezzetti *et al*., 2019). Therefore, with the intensive course of metabolic processes in highly productive cows, even minor management errors on farms cause the development of morbidity, specifically metabolic pathology (Banda *et al*., 2022; Grala *et al*., 2022).

Metabolic diseases of cows are a considerable obstacle to increasing productivity in dairy cattle breeding. They lead to significant economic losses in animal husbandry due to a shortfall in offspring and milk, an increase in the cost of production (Van Saun, 2016).

Metabolic disorders in the body of animals often occur subclinically, and therefore are not established in time by livestock breeders and are not diagnosed by veterinary doctors. The study of the content of metabolic indicators in the body of cows during the transit period should be considered when developing a strategy for monitoring metabolic disorders, which is a component of preventive measures against the spread of relevant diseases and early culling of valuable livestock. Therefore, it is important for specialists working with highly productive dairy cows to consider the patterns of metabolism in the body of healthy animals during the transit period (Smith *et al*., 2017). This is also important for determining effective means of treating sick animals and timely implementation of preventive measures that would prevent the progression of pathology. At the same time, the negative energy balance and metabolic disorders that occur during the transit period contribute to immunosuppression, which can lead to the development of both internal diseases and infectious pathology (Hailemariam *et al*., 2014; Lacassea *et al*., 2018).

Consequently, the state of metabolism in the body of dairy cows during the transit period undergoes significant changes, which are aimed at compensating for increased needs for energy, nutrients, and biologically active substances. The cow's body responds to the negative state of energy balance through compensating for the lack of energy by mobilizing carbohydrates, proteins, and lipids of the body depot (Weber *et al*., 2013). The conducted literature analysis and earlier studies by authors of the present paper indicate that it is currently important to conduct medical examinations or monitoring studies in dairy farms to establish the main blood parameters that characterize the state of metabolism in cows during the transition period (Simonov & Vlizlo, 2015; Walter *et al*., 2022).

The purpose of this study was to find the concentration of glucose, pyruvate, and lactate in the blood plasma of dairy cows before calving and in the first weeks of lactation, as well as hormonal regulation and mechanisms of metabolic disorders during this period.

Materials and Methods

The study was carried out per the provisions of the "European Convention for the protection of vertebrates used for experimental and other purposes" (Strasbourg, 1986) (European convention..., 1986) and considering the "General Ethical Principles of Animal Experiments" (Galkin & Grigorenko, 2011), which were adopted by the first National Congress on Bioethics (Ukraine, 2001).

The cows were studied on the farm of the Lviv region, and blood tests were carried out in the laboratory of Molecular Biology and Clinical Biochemistry of the Institute of Animal Biology of the National Academy of Agrarian Sciences and at the Department of Internal Diseases of Animals and Clinical Diagnostics of the Stepan Gzhytskyi National University of Veterinary Medicine and Biotechnologies Lviv during 2020-2021. The object of this study was highly productive cows of the Holstein breed, at various stages of the physiological state and periods of maintenance. The first period of the study concerned cows 7-10 days before calving, the other three periods – after calving: the second – Days 2-4, the third – Days 10-14, and the fourth – Days 30-40. Analogous cows were selected for the study in terms of productivity, body weight, and age. Cows corresponded to 2-4 lactations, with a productivity of 7.8-8.2 thousand kg of milk per previous lactation. Overall, the study was conducted on 80 dairy cows. All animals underwent a daily clinical examination. If sick animals were identified, they were prescribed treatment, depending on the diagnosed pathology. Animals that were treated were not used for further studies.

Blood for research was obtained from the jugular vein of cows, in the morning, before feeding them. The blood was stabilized by adding heparin to the test tubes. After taking blood from cows, plasma was obtained directly on the farm. For this, the blood was immediately centrifuged at 3,000 rpm for 20 minutes. The resulting blood plasma was transferred to chemically clean, dry test tubes, and transported in a portable refrigerator to the laboratory, where it was stored in a stationary refrigerator at +4-6°C and used for analysis (up to three days). The rest of the obtained blood plasma was frozen at -20°C and stored for up to two months before the next analysis. In the blood plasma of cows, glucose concentrations were determined using the glucose oxidase method, pyruvate – according to the modified Umbright method, and lactate – by reaction with paraoxydyphenyl. These studies were performed on a biochemical Evolution 3000 analyser (Italy). The concentration of hormones in the blood plasma (insulin, cortisol, insulin-like growth factor, leptin, thyroid-stimulating hormone, triiodothyronine, thyroxine) was established according to the enzyme immunoassay method using test kits from the companies "Human" (Germany), "DRG" (Germany), and "Orgentec" (Netherlands).

Statistical analysis of blood parameters was performed using a personal computer and Microsoft Excel software. In distinct groups of cows, the arithmetic mean (M) and statistical error of the arithmetic mean (m) were found from the obtained blood parameters. The correlation coefficient (r) was calculated between individual groups and indicators. The probability of differences in values between groups was calculated using the Student's criterion. Differences were considered significant at $P<0.05$-0.001. Diagrams in figures were constructed using Excel according to generally accepted algorithms. The figures in this paper contain average values of quantities (M).

Results and Discussion

The concentration of glucose in the blood is the main indicator according to which the level of energy supply to the body is determined. Studies have proved that the low concentration of glucose in the blood plasma of cows (Fig. 1) is recorded in the last 7-10 days before calving (2.5 ± 0.2 mmol/L), while on Days 2-4 and 10-14 after calving, its concentration was marked by the lowest values (2.2 ± 0.1 mmol/L and 2.2 ± 0.1 mmol/L, respectively).

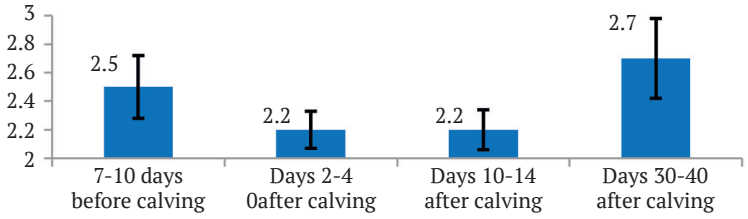

Figure 1. Concentration of glucose in the blood plasma of cows during the transit period, mmol/L

Low plasma glucose concentrations in cows are usually recorded during the first weeks after calving (Vossebeld *et al.*, 2022). Such changes can be considered as a result of a mismatch between energy intake with feed and glucose consumption for metabolic processes and milk synthesis. In healthy, highly productive cows, the need for energy and protein in the first days after calving exceeds their consumption with feed. Therefore, with a shortage of glucose, cows use their lipid reserves from the depot of the body, which causes an increase in the level of non-esterified fatty acids and ketone bodies in the blood (Imhasly *et al.*, 2015). Such changes can cause metabolic disorders, specifically the development of ketosis. Furthermore, glucose is the best fuel for immune cells, so its low concentration during the transition period and high content of ketone bodies can cause immunosuppression (Ingvartsen & Moyes, 2013) and weaken the body's defences.

On Days 30-40 after calving, the blood glucose content of cows increased to 2.7 ± 0.3, or 23% compared to the first two weeks after calving (Fig. 1). However, in another 40% of animals, its level stayed below the lower limit of physiological fluctuations (2.5-3.5 mmol/L).

Glucose metabolism in body cells depends on access to oxygen. During the oxidation of glucose under aerobic conditions, pyruvate is formed in the body, and when the breakdown occurs without oxygen, glycolysis occurs anaerobically with enhanced formation of lactate. It was found that simultaneously with a decrease in the concentration of glucose in the blood plasma of cows after calving, the content of pyruvate and lactate increases (Figs. 2 and 3), which indicates the activation of metabolic processes both by aerobic and anaerobic pathways.

Thus, on Days 2-4 after calving, the content of pyruvate in the blood plasma of cows was 114.9 ± 4.8 μmol/L, and lactate – 1.4 ± 0.3 mmol/L, which is 8% and 50% higher, respectively, compared to the period before calving (106.2 ± ± 4.01 μmol/L and 0.9 ± 0.2 mmol/L, respectively).

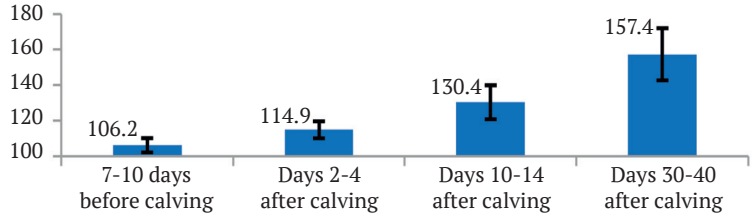

Figure 2. Concentration of pyruvate in the blood plasma of cows during the transit period, μmol/L

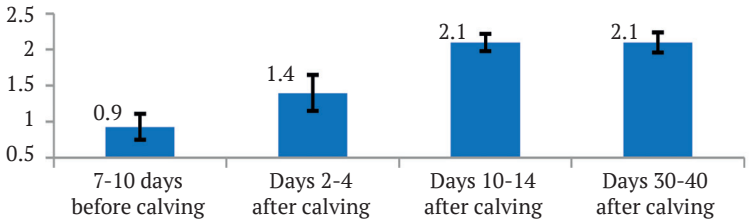

Figure 3. Concentration of lactate in the blood plasma of cows during the transit period, mmol/L

Pyruvate and lactate values increased even more at Days 10-14 and 30-40 of lactation. During these study periods, the concentration of pyruvate in the blood plasma of cows exceeded its value in the prenatal period by 23% (130.4 ± 5.3 μmol/L; P<0.01) and 48% (157.4±7.03 μmol/L; P < 0.001), and lactate – by 2.3 times (2.1 ± 0.3 and 2.10 ± 0.5 mmol/L; P < 0.001), respectively. Notably, after calving, the ratio of lactate to pyruvate in the blood of cows was consistently higher than that of dry cows (Fig. 4), which may indicate an increase in gluconeogenesis processes. Thus, if 7-10 days before calving the ratio of lactate to pyruvate corresponded to 9, then on Days 2-4 after calving it corresponded to 12, on Days 10-14 – to 17, and on Days 30-40 days – to 14.

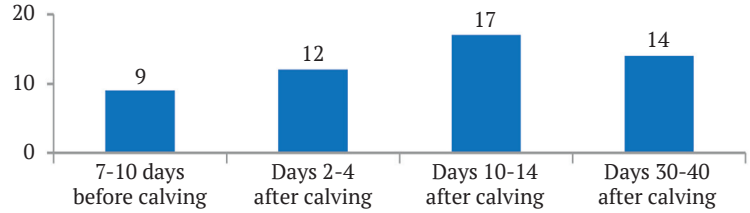

Figure 4. Ratio of lactate to pyruvate in cow blood plasma during the transit period

The metabolism of carbohydrates in the body of ruminants is aimed at maintaining the stability of the concentration of glucose in the blood, including during energy deficit, since the brain feeds exclusively on glucose. Proceeding from this, at the beginning of lactation, when the need for glucose for milk lactose synthesis is high and the supply of glucose precursors from feed is low, activation of gluconeogenesis is a vital compensatory mechanism (Schulz *et al.*, 2014). In ruminants, to provide the body with energy, gluconeogenesis is continuous and increases after feeding. Their need for glucose is provided as a result of gluconeogenesis by 90% or more, which is regulated by the endocrine system (Knob *et al.*, 2021). Thus, in the blood plasma of cows on Days 2-4 after calving, a decrease in the concentration of insulin to 122.8 ± 16.5 pmol/L (P < 0.001) was found compared to 260.9 ± 19.2 pmol/L in the period of 7-10 days before calving (Fig. 5). On Days 10-14 and 30-40 after calving, the plasma insulin concentration was 163.5 ± 17.7 and 164.6 ± 15.8 pmol/L, which was 33% and 34% higher, respectively, compared to Days 2-4 (122.8 ± 16.5 pmol/L), but even lower by 38% (P < 0.05) and 37%, respectively, than in dry animals (260.9 ± 19.2 pmol/L). Low insulin concentrations lead to inhibition of glycogenesis and contribute to the support of plasma glucose levels (Vossebeld *et al.*, 2022). In the literature, the reduction of insulin synthesis by the pancreas of dairy cows of the transit period is called insulin resistance (Rico *et al.*, 2015). Insulin resistance is a consequence of the body's metabolic adaptation to energy deficiency. This process increases gluconeogenesis in the liver in cows and reduces glucose uptake by skeletal muscles and other tissues (Spachmann *et al.*, 2013).

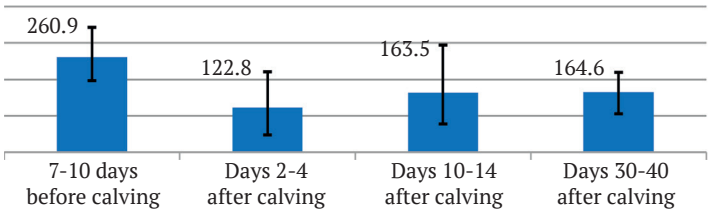

Figure 5. Concentration of insulin in the blood plasma of cows during the transit period, pmol/L

However, insulin resistance accelerates lipolysis during the transition period, and this increases the risk of metabolic diseases in dairy cattle (Rico *et al.*, 2015). Therefore, a decrease in insulin synthesis at the beginning of lactation is probably associated with glucose deficiency and increased gluconeogenesis. Activation of gluconeogenesis increases the mobilization of energy substances from the depot, especially lipids, which can adversely affect the intensity and direction of metabolism and provoke the development of metabolic pathologies (Weber *et al.*, 2013; Gruber & Mansfeld, 2019). Specifically, increased lipomobilization and the development of excessive amounts of free fatty acids causes functional liver overload and the development of fatty hepatocyte infiltration (Ingvartsen & Moyes, 2013; Imhasly *et al.*, 2015).

The next pathway of physiological metabolism regulation occurs through the activation of the adrenal glands, which produce glucocorticoids, primarily cortisol. The latter induces all key enzymes of gluconeogenesis and provides this process with starting compounds and reduces the need for glucose in tissues, thereby increasing its level in the blood (Ebinghaus *et al.*, 2020). In turn, this helps the body overcome stress, support a certain level of glucose in the blood, even with insufficient intake of carbohydrates. By stimulating protein breakdown, cortisol promotes the release of amino acids, which are also essential elements of gluconeogenesis. The conducted studies of cortisol concentration in blood plasma of cows indicated (Fig. 6) its highest level on Days 2-4 and 10-14 of lactation.

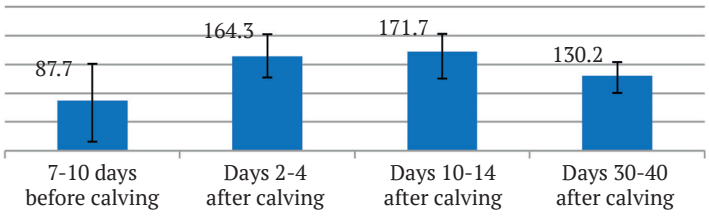

Figure 6. Concentration of cortisol in the blood plasma of cows during the transit period, μIU/L

Thus, the content of cortisol in blood plasma after calving on Days 2-4 was higher by 87% (164.3 ± 17.3 μIU/L; P < 0.01), on Days 10-14 – by 96% (171.7 ± 16.9 μIU/L; P < 0.001), and on Days 30-40 – by 48% (130.2 ± 14.8 μIU/L; P < 0.05) compared to the end of the dry period (87.7 ± 12.4 μIU/L). Increased cortisol synthesis during energy deficiency can enhance gluconeogenesis through lipolysis and proteolysis (Gross *et al.*, 2015).

Apart from insulin and cortisol, markers of metabolism in dairy cows can also be the concentration of insulin-like growth factor (IGF, somatomedin) and leptin in blood plasma (Petruh *et al.*, 2018). Presently, IGF and leptin can be attributed to understudied hormones. Insulin-like growth factor is synthesized mainly in the liver in response to an increase in the level of somatotropic hormone in the blood. In its physiological properties, IGF is close to insulin, structurally similar, and has common receptors that trigger the same reaction chains (Simonov *et al.*, 2021). It stimulates the transport of amino acids and glucose in muscles, increases the sensitivity of cells to insulin, while in adipose tissue it promotes the transport of glucose, its oxidation and transformation into lipids (Mollo *et al.*, 2021). That is why the concentration of IGF decreased in the blood of cows after calving (Fig. 7) compared to the dry period.

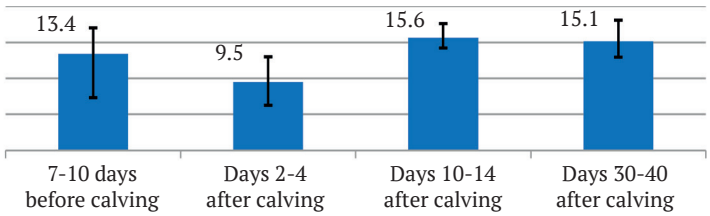

Figure 7. Concentration of insulin-like growth factor (IGF) in cow blood plasma during the transit period, nmol/L

Thus, the concentration of IGF in blood plasma on Days 2-4 days of lactation was 29% lower (9.5 ± 1.5 nmol/L; P < 0.05) compared to the period of 7-10 days before calving (13.4 ± 1.3 nmol/L). The correlation between blood insulin concentrations and IGF during the first day after calving is remarkably high (r = +0.9). Consequently, when dairy cows develop a negative energy balance after calving and a decrease in blood glucose concentration, the endocrine system inhibits the synthesis of insulin, IGF, and glucagon (Vossebeld *et al.*, 2022; Fazio *et al.*, 2022).

On Days 10-14 after calving, compared to Days 2-4, the concentration of IGF in the blood plasma of cows increased by 64% (15.6 ± 1.4 nmol/L; P < 0.01) and stayed at this level until Days 30-40 (15.1 ± 1.7 nmol/L; P < 0.05). However, the correlation between IGF and insulin levels on Days 10-14 after calving was still quite positively high (r = +0.7). This suggests that IGF is important in the energy supply of the body of dairy cows.

The tissue hormone leptin is synthesized by adipocytes, and its main physiological function is to reduce the synthesis of macroergic compounds and increase energy expenditure. Circulating in the blood, leptin helps support optimal glucose levels, which are essential for the body's energy needs (Petruh *et al.*, 2018). The results of the conducted studies indicate (Fig. 8) that, compared to the end of the dry period (8.10 ± 0.76 μg/L), on Days 2-4 of lactation, the content of leptin in blood plasma decreased by three times (2.67 ± 0.33 μg/L; P < 0.001) and tayed at a low level on Days 10-14 (2.22 ± 0.42 μg/L; P < 0.001) and until the Day 40 of lactation (2.30 ± 0.61 μg/L; P < 0.001).

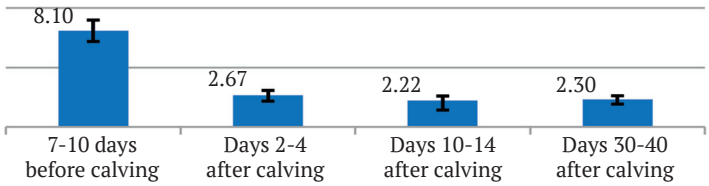

Figure 8. Leptin content in cow blood plasma during the transit period, μg/L

Leptin is involved in the regulation of appetite, interacting with specific receptors located in the hypothalamic region, activates the production of nerve impulses directed to the corresponding areas of the brain. An elevated concentration of leptin in the blood signals that the body has received enough nutrients. Thus, the low content of leptin in the blood plasma of cows after calving established by the results of the study contributes to an increase in feed consumption at the beginning of lactation, when the body's need for nutrients, especially energy, increases considerably.

Thyroid hormones (triiodothyronine, thyroxine) and its main regulator, the pituitary gland, which produces thyroid-stimulating hormone (TSH) play a vital role in the regulation of metabolism in the body of cows, specifically energy metabolism (Fazio *et al.*, 2022). Studies have proved that in comparison with the period of 7-10 days before calving, in the first four days after calving, the concentration of triiodothyronine (T3) decreases (Figs. 9 and 10) in the blood plasma of cows by 54% (from 4.8 ± 0.5 to 2.2 ± 0.3 nmol/L; P < 0.001) and thyroxine (T4) – by 35% (from 80.6 ± 6.3 to 52.2 ± 5.0 nmol/L; P < 0.01).

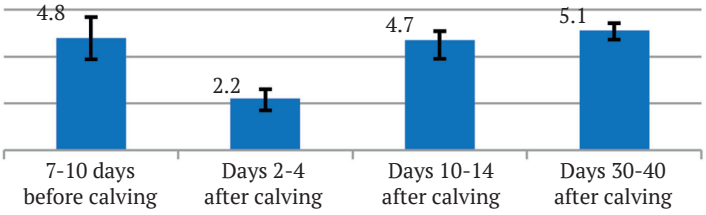

Figure 9. Triiodothyronine concentration (T3) in the blood plasma of cows during the transit period, nmol/L

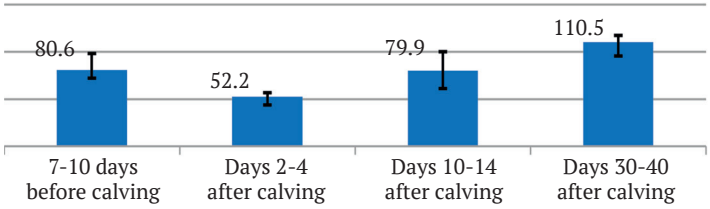

Figure 10. Thyroxine concentration (T4) in the blood plasma of cows during the transit period, nmol/L

At the same time, the study of the concentration of thyroid-stimulating hormone in the blood plasma of cows showed no significant difference (Fig. 11). It was at the same level (0.21 ± 0.031 and 0.2 ± 0.027 mIU/L, respectively, in the pre-calving period and on Days 2-4 after calving).

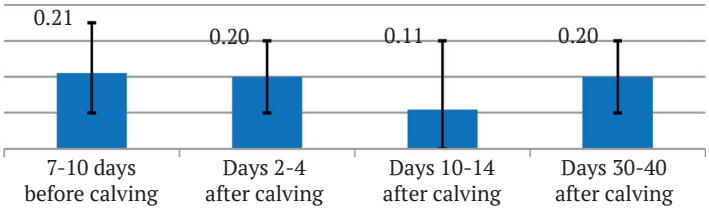

Figure 11. Thyroid-stimulating hormone (TSH) concentration in blood plasma of cows during the transit period, mIU/L

Having conducted the correlation, it was established that on Days 2-4 after calving, it was strongly positive (r = +0.7) between the level of thyroxine and insulin-like growth factor, and moderately positive (r = +0.4) between the concentration of triiodothyronine and IGF. This may indicate regulatory metabolic processes both in the liver, which produces IGF, and in the thyroid gland. The established low level of IGF and thyroid hormones indicates a decrease in the use of energy compounds by body tissues and increases their availability to the mammary gland. This is one of the mechanisms of energy redistribution in favour of milk formation. This suggests that a decrease in the synthesis of IGF and thyroid hormones is a consequence of the physiological regulatory features of this period. It is indicated that the level of IGF in the blood depends on the concentration of thyroid hormones that increase the secretion of IGF by the liver (Petruh *et al.*, 2018). Starting from Days 10-14 after calving, compared to Days 2-4 days, the concentration of T3 in the blood plasma of cows increased by 2.1 times (4.7 ± 0.5 nmol/L; P < 0.001) and T4 – by 53% (79.9 ± 6.2 nmol/L; P < 0.01). On Days 30-40, the concentration of T3 stayed stable (5.1 ± 0.4 nmol/L; P < 0.001), while T4 – increased (110.5 ± 9.7 nmol/L; P < 0.001) and was the highest for all periods under study (Figs. 9 and 10). The growth of thyroid hormones activates the carbohydrate and protein catabolism, stimulates lipomobilization with a negative energy balance. On Days 10-14 after calving, the correlation between the level of IGF and thyroid hormones was weak (r = +0.2-0.3), in contrast to the nature of the correlation between IGF and insulin level (r = +0.7).

Thus, the analysis of the literature and research of the authors of the present paper indicate that in

high-yielding dairy cows in the transit period, the state of metabolism, specifically energy metabolism, should be monitored, since even minor deviations can cause its disruption and the development of diseases.

Conclusions

In highly productive dairy cows, in the last weeks before calving and the first weeks of lactation period, there is a considerable increase in energy metabolism, which is aimed at ensuring high productivity. In the first 14 days after calving, the concentration of glucose in the blood plasma of cows decreases to 2.2 ± 0.1 mmol/L, which indicates a lack of energy in the body. Compared to the pre-calving period, during the first 40 days of lactation, the concentration of pyruvate in the blood plasma of cows increases by 48% (157.4 ± 7.03 μmol/L; P < 0.001), and lactate, even by 2.3 times (2.1 ± 0.5 mmol/L; P < 0.001), which indicates an increase in the breakdown of carbohydrates, specifically by anaerobic means.

Supporting the physiological course of metabolism in the body of cows of the transit period is actively regulated by the endocrine system. With a low level of glucose in the blood plasma of dairy cows in the first weeks of lactation, compared to the period before calving, the body produces 37-47% less insulin (122.8±16.5-164.6±15.8 pmol/L; P < 0.001-P < 0.05) and leptin by 3.0-3.5 times (2.67 ± 0.33-2.30 ± 0.61 μg/L; P < 0.001), and in the first 4 days also insulin-like growth factor by 29% (9.5 ± 1.5 nmol/L; P < 0.05) and increases the synthesis of cortisol from 48% to 96% (130.2 ± 14.8-171.7 ± 16.9 μIU/L; P < 0.05-0.001), and from 10 to 40 days – triiodothyronine (5.1 ± 0.4 nmol/L; P < 0.001) and thyroxine (110.5 ± 9.7 nmol/L; P < 0.001), which contributes to the stabilization of energy metabolism. Therefore, in the transit period, a well-coordinated regulatory action on the part of the endocrine system is important, which would ensure the completion of pregnancy, successful calving, high milk yield due to increased activity and support of metabolic intensity at the physiological level.

Further research will relate to the investigation of metabolic pathology, elucidation of aetiological factors, and pathogenetic mechanisms of metabolic diseases in dairy cows in the first weeks after calving.

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Гормональна регуляція концентрації глюкози та її похідних у крові молочних корів під час транзитного періоду

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Анотація. Порушення обміну речовин у молочних корів переважно реєструють в останні тижні сухостійного періоду та перші – після отелення (транзитний період). Метою роботи було дослідити гормональну регуляцію концентрації глюкози та її похідних у крові молочних корів під час транзитного періоду. Дослідження проводили на коровах 2–4 лактацій, продуктивністю 7,8–8,2 тис. кг молока за попередню лактацію. Кров для досліджень відбирали в корів за 7–10 діб до отелення та на 2–4 добу, 10–14 та 30–40 доби після нього. Концентрацію у плазмі крові глюкози визначали глюкозооксидазним методом, пірувату – модифікованим методом Умбрайта, лактату – за реакцією з параоксидифенілом, а вміст гормонів – методом імуноферментного аналізу. Встановлено, що у високопродуктивних молочних корів під час транзитного періоду відбуваються істотні зміни в обміні вуглеводів та функціонального стану органів і систем, які спрямовані на забезпечення високої продуктивності. Так, у корів упродовж двох тижнів після отелення реєструється гіпоглікемія. Водночас зі зменшенням концентрації глюкози у плазмі крові корів зростає вміст пірувату та лактату, а також відношення лактату до пірувату, що свідчить про посилення глюконеогенезу. Негативний енергетичний баланс та посилення глюконеогенезу призводять до зниження синтез інсуліну та інсуліноподібного фактору росту. Порівняно із завершальним періодом сухостою, на 2–4 добу лактації, концентрація лептину в плазмі крові корів зменшувалася втричі та трималася на низькому рівні до 40 доби лактації. Рівень кортизолу в плазмі крові був найвищим на 2–4 та 10–14 добу лактації. Інтенсивний синтез кортизолу в період енергетичного дефіциту посилює глюконеогенез, що можливе за рахунок ліполізу та протеолізу. У перші доби після отелення у плазмі крові корів зменшувався вміст тироксину та трийодтироніну. Пригнічення продукції тиреоїдних гормонів є наслідком фізіологічних регуляторних особливостей цього періоду. Таким чином, у високопродуктивних корів під час транзитного періоду слід звертати увагу на підтримання життєво важливих функцій організму та їх злагоджену ендокринну регуляцію, що забезпечить фізіологічно збалансований рівень метаболізму, успішне отелення, високу молочну продуктивність та здоров'я тварин

Ключові слова: молочне скотарство, метаболізм вуглеводів, лактат, піруват, гормони

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Micromorphometric characteristics of the adrenal gland in birds

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Abstract. The adrenal gland is a peripheral organ of the endocrine system that directly affects the formation of bird productivity indicators, which is an important characteristic for the development of industrial poultry farming. The purpose of this study was a morphometric assessment of the microstructural components of the adrenal gland of birds of the order Galliformes (domestic quail, chicken, turkey), Anseriformes (Muscovy duck, domestic duck, and goose) and Columbiformes (common pigeon). Anatomical, histological, morphometric, and statistical research methods were used in this study. It was found that the thickness of the adrenal capsule of birds directly depends on their body weight and varies from 10.82 ± 0.56 μm (domestic quail) to 28.53 ± 1.36 μm (domestic turkey). Interrenal tissue compared to suprarenal tissue in Muscovy ducks, common pigeons, domestic ducks, and geese occupies a larger ($P < 0.001$) area of the central zone (3.50, 2.77, 3.10, and 3.11 times, respectively) and peripheral zone (1.27, 2.71, 1.38, and 1.55 times, respectively) of the adrenal gland, which indicates its morphofunctional activity. The area of the venous sinuses in the central zone compared to the peripheral zone of the adrenal gland is larger in domestic quail by 2.80 times ($P<0.05$), domestic chicken – by 3.62 times ($P < 0.05$), domestic turkey – by 3.68 times ($P < 0.05$), domestic ducks – by 5 times ($P < 0.01$), domestic ducks – by 3 times ($P < 0.05$), domestic geese – by 2 times ($P < 0.05$). The common pigeon is characterized by a uniform placement of venous sinuses along the entire periphery of the adrenal gland and, as a result, similar indicators of their area in the peripheral and central zones. The index of the nuclear-cytoplasmic ratio of endocrinocytes of the adrenal gland of birds varies. It is the lowest in the cells of the second type of interrenal tissue (from 0.052 ± 0.004 in the common pigeon to 0.092 ± 0.016 in the domestic quail), slightly higher in the cells of the first type of interrenal tissue (from 0.065 ± 0.004 in the common pigeon to 0.111 ± 0.012 in the domestic turkey) and the largest in chromaffin cells of the supranarial tissue (from 0.102 ± 0.015 in the domestic chicken to 0.166 ± 0.018 in the common pigeon). It is recommended to use the established features of the morphometric indicators of the microstructural components of the adrenal gland of birds to create a base for its normal morphological characteristics. This allows assessing the morphological and functional state of the adrenal gland under several factors and pathology

Keywords: adrenal zones, interrenal and suprarenal tissues, venous sinuses, endocrinocytes, nuclear-cytoplasmic ratio

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Introduction

Birds are a large class of vertebrates. Presently, about 10 thousand bird species from 27 genera and 170 families are known. Among them, over 5 thousand birds are songbirds. The total number of birds reaches 100 billion specimens. The largest number of species is recorded in countries such as Colombia (1,700), Ecuador (1,357), and Brazil (1,440). A much smaller number of bird species is typical for Cameroon (670), the USA and Canada (775), and very few – for Portugal (315) and Greece (339) (Batool *et al*., 2020; Lees *et al*., 2022).

Over many centuries, humans have domesticated several Galliformes – turkeys, guineafowl, chickens, quails, pheasants, peacocks; of Anseriformes – ducks, geese, mute swan; of Columbiformes – common pigeon. Man has learned to breed canaries, ostriches, cormorants, parrots, and other birds in captivity (Domyan & Shapiro, 2017).

Industrial poultry farming occupies a leading position among livestock industries (Hafez & Attia, 2020). At the beginning of the 21st century, the production of eggs and poultry meat increased fivefold (OECD-FAO Agricultural..., 2016). According to the OECD-FAO agricultural forecast for 2019-2028, poultry is the most consumed animal protein in the world (OECD-FAO Agricultural..., 2019).

The main area of development of modern poultry farming and veterinary medicine is to improve the productive qualities of birds and prevent diseases of various origins (Rudik *et al*., 2021). Organizational and economic activities measures require an in-depth study of the bird body in general and the morphology of organs and systems in particular. Morphometric studies of endocrine organs in clinically healthy birds at the tissue and cellular levels are an urgent issue, since they can be used as morphological criteria for diagnosing diseases in humans and animals. The high efficiency of morphometric assessment of the state of the animal body at the organ, tissue, and cellular levels has been proven (Shevchenko, 2018).

The endocrine system, together with the nervous system, coordinates the activity of the entire body (Lotfi, 2018). According to the modern classification, the organs of the endocrine system are divided into three groups: central, peripheral, and mixed. They also include single endocrinocytes. They are located in non-endocrine organs, forming a dissociated endocrine system (Scanes, 2015).

An essential role in the functioning of the endocrine system of birds belongs to the adrenal gland. Its hormones affect tissue differentiation and growth, the development of reproductive organs, and the course of the sexual cycle (Banerjee, 2018; Kot *et al*., 2021; Gjuen, 2022). They regulate water, protein, carbohydrate, fat and mineral metabolism (Lotfi *et al*., 2018; Di Lorenzo, 2020). The adrenal gland is sensitive to changes in the internal and external environment. Stress, hypothermia, and diseases change the mass, size, vascularization of the adrenal glands, the ratio of cortical and cerebral zones, and their cytophysiological characteristics (Lotveld *et al*., 2017; Qureshi *et al*., 2020).

In birds, compared to mammals, the microscopic structure of the adrenal gland has class features of the histoarchitectonics of its structural components. Externally, the adrenal gland of birds is covered with a connective tissue capsule, under which the parenchyma is located. The latter is formed by suprarenal and interrenal tissues, which are represented by cell cords that are chaotically intertwined. The different configuration of cell cords of interrenal and suprarenal tissues allows conditionally distinguishing two zones – peripheral and central – on the section of the adrenal gland of birds. The narrow spaces between the cellular cords of the suprarenal and interrenal tissues are filled with loose fibrous connective tissue with venous sinuses and sinusoidal hemocapillaries (Kober *et al*., 2012; Matos, 2008; Colcimen & Carmak, 2020; Prokopenko & Kot, 2021).

Morphology uses a variety of research methods: anatomical, pathoanatomical, histological, immunohistochemical, ultramicroscopic, histochemical, and morphometric. The latter method is characterized by high accuracy for assessing the structural and functional state of animals at the levels of organs, tissues, and cells in normal and pathological conditions (Bancroft & Gamble, 2007). Scientific sources mainly present information on organometry of the adrenal gland of birds (Kober *et al*., 2012; Colcimen & Cakmak, 2020; Prokopenko & Kot, 2021). Information on the morphometry of microstructural components of the adrenal glands in birds is limited. Most authors investigated one to two adrenal micromorphometric parameters in birds of certain species and age groups. Specifically, Fathima & Lucy (2014) established an inter-suprarenal ratio of the adrenal glands in domestic ducks. Matos (2008) determined the height of endocrinocytes and the diameter of their nuclei in this gland in domestic chicken. Moghadam & Mohammadpour (2017) specified the relative volume of the interrenal and suprarenal tissues of the adrenal gland in domestic guineafowls. El-Desoky & El-Zahraa (2021) noted the area of interrenal and suprarenal adrenal tissues in domestic quail. There are no data from a comprehensive morphometric study of the microstructural components of the adrenal gland of birds in the comparative-species aspect. The issue of cytomentric parameters of adrenal endocrinocytes in birds is not covered.

Therefore, the purpose of this study was a comprehensive morphometric assessment of the microstructural components of the adrenal gland of birds of various species, the indicators of which are morphological criteria of physiological and pathological changes in the endocrine system and can be used during the diagnosis of diseases of various aetiology.

To fulfil the purpose of the study, it was necessary to solve the following tasks after anatomical and histological examination of the adrenal gland: to establish the thickness of the adrenal capsule; to find the area of suprarenal, interrenal tissues, and venous sinuses in the peripheral and central zones of the adrenal gland; to conduct morphometric analysis of adrenal endocrinocytes.

Literature Review

The specialized literature of the last five years presents information on the morphology of the adrenal gland of fish, amphibians, reptiles (Gaber & Abdel-maksoud, 2019; Di Lorenzo *et al*., 2020) and mammals (Kigata & Shibata, 2018; Zakrevska & Tybinka, 2019). Regarding birds, most of the studies by ornithologists and morphologists provide data on the anatomical and microscopic structure of the adrenal gland of various bird species (Qureshi *et al*., 2020; Prokopenko & Kot, 2021). The adrenal gland is a parenchymatous organ, the microscopic structure of which corresponds to the regularities of the structure and function of endocrine

organs. There are no excretory ducts in the endocrine organs. The products of their activity (hormones) are released directly into the lymph and blood. In terms of the structure, endocrine organs consist of connective tissue stroma and parenchyma. The latter forms follicles, rods, islands, the cells of which are in close contact with the vessels of the microcirculatory channel. There are many blood capillaries between the structural components of the adrenal parenchyma, mainly of the sinusoid type (Scanes, 2015; Kigata & Shibata, 2018; Zakrevska & Tybinka, 2019). Externally, the adrenal gland of birds is covered with a capsule, which is formed by loose fibrous connective tissue, contains blood vessels and clusters of nerve cells. In the common pigeon, nodes of the autonomic nervous system are also registered in it, in the domestic chicken – supplementary adrenal glands.

In birds of the Anseriformes order, the outside of the capsule of the adrenal gland contains a layer of adipose tissue, in Muscovy duck – a group of nodes of the autonomic nervous system (Kot *et al*., 2021; Prokopenko & Kot, 2021). The results of the morphometric study of the macro- and microscopic components of the adrenal gland of birds are incomplete, scattered, and refer to individual species. There is evidence that the mass, length, thickness, and width of the right and left adrenal glands of birds are not the same. In domestic birds, the left adrenal gland has a larger mass (Prokopenko & Kot, 2021). The absolute mass index of the right and left adrenal glands of domestic chicken is 97.2 ± 1.2 and 104.1 ± 1.4 mg; length – 0.8 ± 0.0 and 0.9 ± 0.0 cm; width – 0.6 ± 0.0 and 0.5 ± 0.0 cm; thickness – 0.5 ± 0.0 and 0.4 ± 0.0 cm, respectively (Kober *et al*., 2012).

According to Fathima *et al*. (2014), adrenal gland mass is more correlated with bird age than with body weight. In common pigeon (*Columba livia*), house sparrow (*Passer domesticus*), house crow (*Corvus splendens*), common myna (*Acridotheres tristis*), bank myna (*Acridotheres ginginianus*), black kite (*Milvus migrans*), grey francolin (*Francolinus pondicerianus*), and in western cattle egret (*Bubulcus ibis*), the mass of adrenal glands increases during the period of their sexual activity (Vyas & Jacob, 1976). In addition, the organometric indicators of the adrenal gland of birds are affected by their growing conditions, stress, and the effect of pharmacological preparations (Qureshi *et al*., 2020; Rudik *et al*., 2021).

In terms of the morphometric parameters of the microstructural components of the adrenal gland of birds, the ratio between the interrenal and suprarenal tissues is determined by the species, age, and sex of the birds. According to Kober *et al*. (2012), in the adrenal gland of domestic chicken, the proportion of supraranal tissue in the central zone (49.7%) is twice as high as in the peripheral zone (24.8%). In general, the inter-supraranal ratio is 1.6:1. In the domestic duck, the inter-suprarenal ratio of the adrenal gland changes from 1.15:1 (one day old) to 2:1 (24 weeks old) due to an increase in the proportion of interrenal tissue in the gland (Fathima & Lucy, 2014). In domestic chicken, the inter-suprarenal ratio in males and females is not the same – 1.9:1 and 1.43:1, respectively. In a 35-day-old domestic quail, this indicator is 1:1, and by the 55th day it changes to 6:1 (Matos, 2008).

Morphological analysis of the cells of the adrenal parenchyma of birds shows that the endocrinocytes of the suprarenal tissue have a basophilic cytoplasm, a polygonal shape, and a rounded nucleus located in the centre (Vyas & Jacob, 1976; Prokopenko & Kot, 2021). In domestic chickens, the height of cells and the diameter of their nuclei in the adrenal glands of males (8.72 ± 0.231 μm and 4.39 ± 0.359 μm, respectively) are greater than in females (8.67 ± 0.218 μm and 3.84±0.326 μm, respectively) (Matos, 2008). Cells of the interrenal adrenal tissue of birds have an eosinophilic stained cytoplasm, columnar or cubic shape, rounded or oval nucleus, which is located eccentrically (Fathima & Lucy, 2014; Prokopenko & Kot, 2021). In domestic chickens, the height of cells and the diameter of their nuclei in the adrenal glands of males (8.96 ± 0.159 μm and 4.26 ± 0.166 μm, respectively) are smaller than in females (10.57 ± 0.628 μm and 3.69 ± 0.087 μm, respectively) (Matos, 2008).

Materials and Methods

The study was conducted during 2019-2022 in the Educational and Scientific Clinical and Diagnostic Laboratory of the Faculty of Veterinary Medicine of the Polissia National University (Zhytomyr). The study performed is a fragment of the research "Morphology of the adrenal gland of birds" (State Registration No. 0120U101089). The adrenal gland was collected from sexually mature birds of such species as domestic quail (*Coturnix coturnix. var. domesticus*), domestic chicken (*Gallus gallus. var. domesticus*), domestic turkey (*Meleagris gallopavo. var. domesticus*), domestic duck (*Anas platyrhynchos. var. domesticus*), Muscovy duck (*Cairina moschata*), domestic goose (*Anser anser. var. domesticus*) and common pigeon (*Columba livia*).

The test birds were clinically healthy and showed no signs of disease. All intervention and slaughter of birds was carried out in compliance with the recommendations of Directive 2010/63/EU of the European Parliament and the Council of September 22, 2010 "On the Protection of animals used for Scientific Purposes" (Directive 2010/63/EU..., 2010) and accordingly to the Law of Ukraine No. 692 "On the Protection of Animals from Cruel Treatment" (3447-IV) dated February 21, 2006 (On the Protection..., 2006).

Morphometric studies of the adrenal glands of birds were preceded by anatomical and histological studies. Anatomical studies included weighing of birds, their slaughter and exsanguination, dissection of the chest cavity, preparation of the adrenal gland and its subsequent removal (Reavill & Schmidt, 2019). Avian euthanasia included an inhaled overdose of chloroform followed by acute exsanguination due to subclavian artery incision (Brooks Brownlie & Munro, 2016). Body weight of birds (233.17 ± 3.84 g – domestic quail, 1,703.33 ± 37.74 g – domestic chicken, 4,330.00 ± 67.18 g – domestic turkey, 2,145.17 ± 16.86 g – Muscovy duck, 2,492.50 ± 12.23 g – domestic duck, 3,147.50 ± 26.13 g – domestic goose, 327.00±13.28 g – common pigeon) was determined by weighing on a PS6000/C/2 scale (Poland). For histological examination, the adrenal glands of birds were fixed in a 10% aqueous neutral solution of formalin, gradually dehydrated in 40°, 70°, 96° and 100° ethyl alcohol, compacted in one portion of alcohol-xylene in a 1:1 ratio and two servings of xylene, poured into paraffin at a temperature not exceeding 60°C. Histological sections 5-8 μm thick were made from paraffin blocks on a sled microtome MC-2 (Ukraine). They were placed on slides and stained with hematoxylin and eosin (Mulisch & Welsch 2015; Bancroft & Gamble, 2019).

Morphometric methods were used to obtain objective morphometric parameters of the microstructural components of the adrenal glands in birds. For this, the authors of this study used the Aperio ImageScope software (Leica Biosystem Inc. USA, 2021). The following morphometric parameters were determined: capsule thickness, area of supreranal and interrenal tissues, venous sinuses, volume of endocrinocytes and their nuclei, nuclear-cytoplasmic ratio. These morphometric parameters were determined on 5 preparations from each bird, in 10 fields of view of a Primo Star microscope (Carl Zeiss, Germany).

Since the endocrinocytes of the adrenal gland of birds have a shape close to an ellipsoid, the Equation 1 of W. Jacobi was used to determine the volume of these cells and their nuclei (Bancroft & Gamble, 2018).

$$V = \frac{\pi}{6} \times A \times B^2 \quad (1)$$

where V is the cell volume;
π is 3.14; A – large diameter;
B – small diameter.

The nuclear-cytoplasmic ratio is the main cytomorphometric indicator of the level of cell differentiation and metabolism in animals, depending on their living conditions (Gaber & Abdel-maksoud, 2019). Therefore, thanks to this indicator, it is possible to establish the functional activity of adrenal endocrinocytes. The nuclear-cytoplasmic ratio was determined according to the Equation 2:

$$NCR = \frac{Vnucleus}{Vcells - Vnucleus} \quad (2).$$

Digital data of morphometric studies were processed using the variational statistical method using the Statistica 6 software package (Stat Soft Inc., USA). The analysis of the obtained data was based on indicators of descriptive statistics, specifically the arithmetic mean (M) and the standard error of the mean (m). The reliability of the results obtained was evaluated according to the Fischer F-criterion. The difference between the two values was considered significant at $P < 0.05$; $P < 0.01$; $P < 0.001$.

This study is a continuation of a series of works investigating the features of the morphology of the adrenal glands of sexually mature birds in the species aspect (Kot *et al.*, 2021; Prokopenko & Kot, 2021). Preliminary studies were conducted on the topography, shape, consistency, and colour of the adrenal gland of birds; determination of the mass, length, thickness, and width of the adrenal gland of birds; clarification of the features of the microscopic structure of the adrenal gland of birds.

Results and Discussion

Analysis of morphometric studies has proved that the thickness of the adrenal capsule of chickens directly depends on their body weight. The greatest absolute mass of the adrenal gland is typical for the domestic turkey (28.53 ± 1.36 μm), slightly smaller – for the domestic chicken (20.12 ± 1.41 μm), and the smallest – for the domestic quail (10.82 ± 0.56 μm). An analogous dependence of this indicator on body weight was recorded in birds of the Anseriformes order. The thickness of the capsule of the adrenal gland of the domestic goose (27.0 ± 0.89 μm) is the largest, the duck (21.30 ± 1.09 μm) is the smallest, and the domestic duck (24.82 ± 0.51 μm) is intermediate. In the common pigeon (Columbidae order), the thickness of the adrenal capsule occupies an intermediate position among all the birds under study and is 13.46 ± 0.67 μm (Fig. 1).

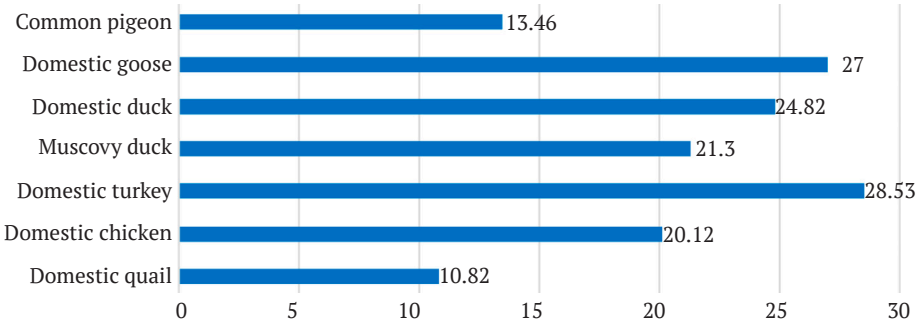

Figure 1. Capsule thickness (μm) of the adrenal gland of birds

The parenchyma of the adrenal gland of birds is formed by cell cords of suprarenal and interrenal tissues that are closely intertwined. Between the cords of these tissues, layers of loose fibrous connective tissue with venous sinuses and sinusoidal hemocapillaries are observed (Prokopenko & Kot, 2021; Erdem *et al.*, 2021). The specific features of the location and configuration of these cell cords are determined by the formation of two zones of the adrenal gland parenchyma of the birds under study – peripheral and central, which confirms the research of other authors who investigated the adrenal gland of the domestic goose (Ye *et al.*, 2018; Grymak *et al.*, 2020). H. Kober *et al.* (2012), investigating the adrenal gland of a domestic chicken, recorded the peripheral and central zones, as well as the subcapsular layer, which corresponds to the research by Fathima and Lucy (2014) in the domestic duck. In the adrenal gland of the ostrich, the central and peripheral zones are distinguished. The latter consists of the outer (subcapsular zone) and inner parts (Ye *et al.*, 2018).

The morphometric study allowed establishing that in the peripheral zone of the adrenal gland, the area of the interrenal tissue compared to the area of the suprarenal tissue is significantly larger in all species of birds under study. Specifically, in domestic quail – by 2.65 times, $P < 0.001$ (71.67 ± 2.93 vs. 27.0 ± 3.17%); domestic chickens – by 2.66 times, $P<0.001$ (71.87 ± 3.62 vs. 27.01 ± 2.64%); turkeys – by 2.85 times, $P < 0.001$ (73.22 ± 5.12 vs. 25.67 ± 4.18%); Muscovy duck – by 3.5 times, $P < 0.001$ (77.0 ± 4.95 vs.

22.0 ± 3.17%); domestic duck – 3.10 times, P < 0.001 (74.5 ± ± 3.54 vs. 24.0 ± 3.65%); domestic goose – 3.11 times, P < 0.001 (74.17 ± 4.09 vs. 23.83 ± 4.35%); grey pigeon – 2.77 times, P < 0.001 (71.5 ± 3.46 vs. 25.83 ± 3.51%). Such ambiguous morphometric indicators of the interrenal and suprarenal tissues of the adrenal gland of experimental birds are consistent with the results of the study of its inter-suprarenal ratio by other researchers (Vyas & Jacob, 1976; Matos, 2008; Grymak *et al*., 2020). According to (Matos, 2008), the inter-suprarenal ratio of the adrenal gland of birds can vary widely from 2:1 (domestic duck) to 6:1 (domestic quail). Venous sinuses occupy the smallest part of the peripheral zone of the adrenal gland of the birds under study. The indicator of their area varies in the birds of the Galliformes order – from 1.12 ± 0.47% (domestic chicken) to 1.33±0.33% (domestic quail), in the birds of the Anseriformes order – from 1.0 ± 0.26% (Muscovy duck) to 2.0 ± 0.63% (domestic goose). In common pigeon, the value of this indicator is the highest among the birds under study and is 2.67 ± 0.33% (Fig. 2).

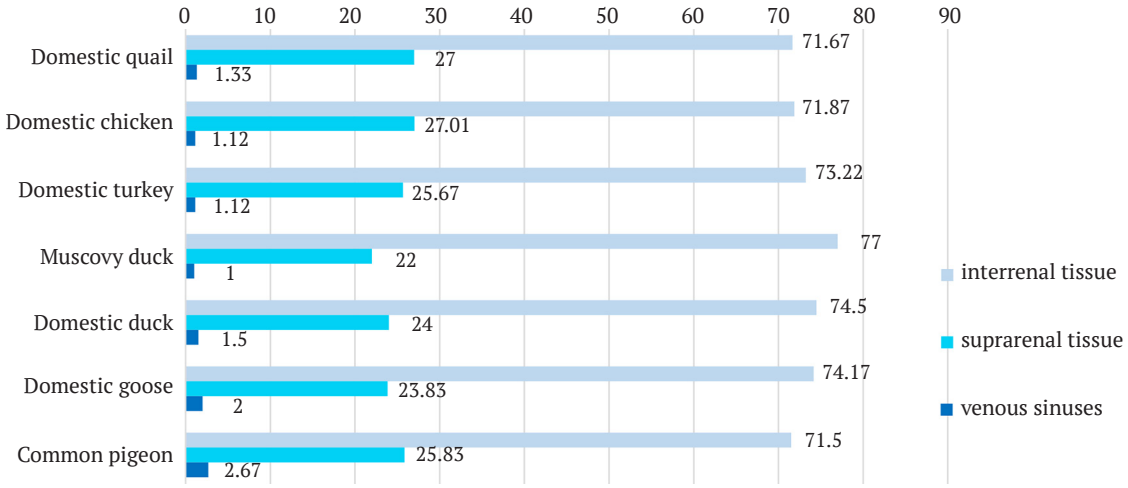

Figure 2. Area (%) of structural components of the peripheral zone of the adrenal gland of birds

In the central zone of the adrenal gland compared to the peripheral zone of domestic quail, chicken, turkey, duck, goose, and Muscovy duck, a significant (P < 0.001) decrease in the area of the interrenal tissue was registered by 1.43, 1.50, 1.44, 1.35, 1.35, and 1.45 times, respectively, to 50.21 ± 1.85%, 48.09 ± 2.03, 50.83 ± 2.30, 55.0 ± 4.68, 58.33 ± 3.07, and 53.08 ± 3.82%, respectively. The indicator of the area of suprarenal tissue of birds of these species, on the contrary, significantly increased (P < 0.001) to 46.88 ± 4.75%, 47.86 ± 2.83, 45.05 ± 2.40, 40.5 ± 5.03, 37.67 ± 2.86 and 41.92 ± 3.37%, respectively. As a result, in birds of the Galliformes order, interrenal and suprarenal tissues occupy a relatively equal share of the central zone of the adrenal gland.

In Anseriformes birds, the area of interrenal tissue is significantly (P < 0.001) larger than the area of suprarenal tissue by 1.27 times (Muscovy duck), 1.38 times (domestic duck), and 1.55 times (domestic goose), which indicates a greater morphofunctional activity of interrenal tissue in this area of the adrenal gland. In common pigeon, the area of the interrenal and suprarenal adrenal tissues in the central zone (71.0 ± 3.50 and 26.17 ± 3.56%, respectively) does not significantly differ from that in the peripheral zone (71.50 ± 3.46 and 25.83 ± 3.51%, respectively) (Fig. 3).

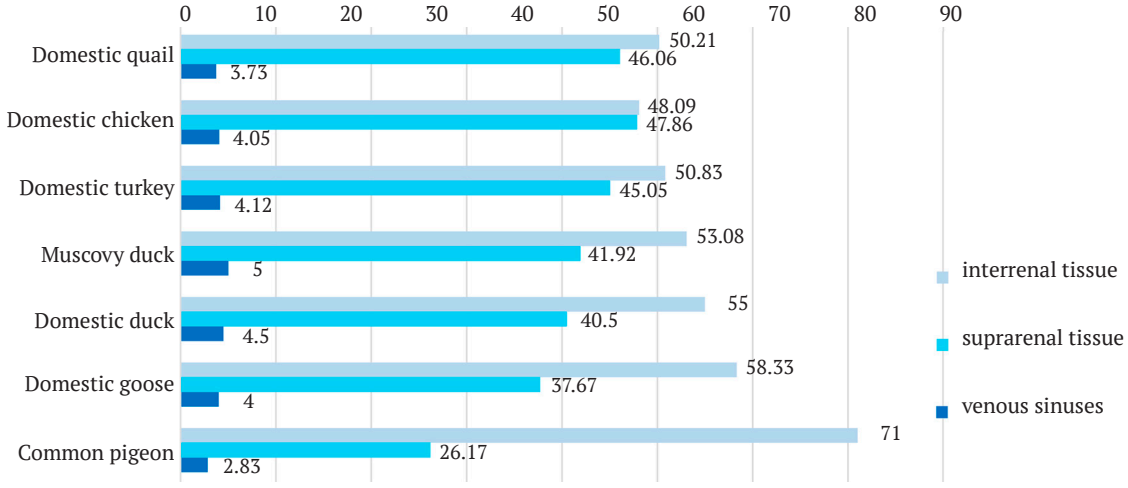

Figure 3. Area (%) of structural components of the central zone of the adrenal gland of birds

In terms of venous sinuses, the indicator of their area in the central zone compared to the peripheral adrenal gland increases in most of the species of birds under study. Specifically, in domestic quail – by 2.80 times (3.73 ± 0.25%, $P < 0.05$), in domestic chickens – by 3.62 times (4.05 ± 0.12%, $P < 0.05$), turkeys – 3.68 times (4.12 ± 0.51%, $P < 0.05$), Muscovy ducks – 5 times (5.0 ± 0.52%, $P < 0.01$), domestic ducks – 3 times (4.5 ± 0.46%, $P < 0.05$), domestic goose – 2 times (4.0 ± 0.26%, $P < 0.05$). In common pigeon, there was no significant difference between the area of the venous sinuses of the central (2.83 ± 0.48%) and peripheral (2.67 ± 0.33%) zones of the adrenal gland (Figs. 2, 3), which indicates the uniform placement of this microstructure in the adrenal parenchyma (Kot *et al.*, 2021; Erdem *et al.*, 2021).

According to the special literature on morphological characteristics of adrenal parenchyma cells in domestic goose (Prokopenko & Kot, 2021), endocrinocytes of suprarenal and interrenal tissues differ in shape, location of the nucleus, and colour of the cytoplasm. The former have a basophilic cytoplasm, while the latter have an acidophilic one. The same two types of cells (basophilic and acidophilic, respectively) were differentiated in domestic ducks by other researchers (Fathima & Lucy, 2014), which contradicts the data of Matos (2008) on four types of cells of the interrenal adrenal tissue of domestic ducks and guineafowl.

To assess the morphological and functional state of the cellular component of the adrenal parenchyma of birds, the volume of endocrinocytes of interrenal and supraranal tissues was found (Figs. 4, 5). According to the obtained digital data, interrenal tissue cells can be divided into two types. Cells of the first type were placed in the peripheral zone, cells of the second type – in the central zone of the adrenal gland of birds. The volume of cell nuclei of these two types probably did not differ and ranged within 11.83 ± 0.65-14.0 ± 1.03 μm³ (domestic quail), within 18.50 ± 1.12-18.66 ± 1.5 μm³ (domestic chicken), within 20 ± 1.69-22 ± 1.13 μm³ (domestic turkey), within 19 ± 1.86-20.0 ± 1.29 μm³ (Muscovy duck), within 19.83 ± 1.08-20.17 ± 1.05 μm³ (domestic duck), within 22 ± 2.35-23.17 ± 1.94 μm³ (domestic goose), within 20.17 ± 1.25-21.33 ± 1.82 μm³ (common pigeon). However, the indicator of the volume of cells of the second type compared to the volume of cells of the first type was significantly greater in all birds under study. Specifically, in domestic quail – by 1.31 times, $P < 0.001$ (171.82 ± 15.12 vs. 131.0 ± 16.02 μm³); domestic chicken – by 1.19 times, $P < 0.01$ (249.5 ± 16.53 vs. 209.67 ± 24.11 μm³); turkey – by 1.43 times, $P < 0.001$ (286.0 ± 13.65 vs. 200.17 ± 13.34 μm³), Muscovy duck – by 1.32 times, $P < 0.001$ (265.67 ± 33.7 vs. 200.67 ± 18.92 μm³), domestic duck – by 1.14 times, $P < 0.01$ (299.83 ± 25.08 vs. 262.67 ± 24.06 μm³), domestic goose – by 1.22 times, $P < 0.001$ (367.67 ± 38.72 vs. 300.33 ± 23.33 μm³), common pigeon – 1.17 times, $P < 0.001$ (401.17 ± 22.15 vs. 342.33 ± 13.80 μm³).

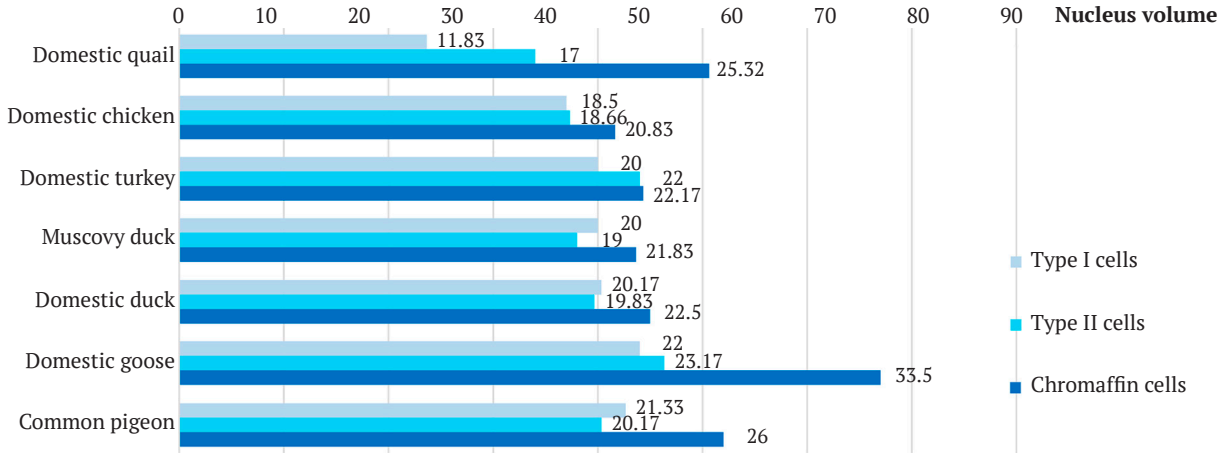

Figure 4. The volume of the nucleus (μm³) of endocrinocytes of the adrenal gland of birds

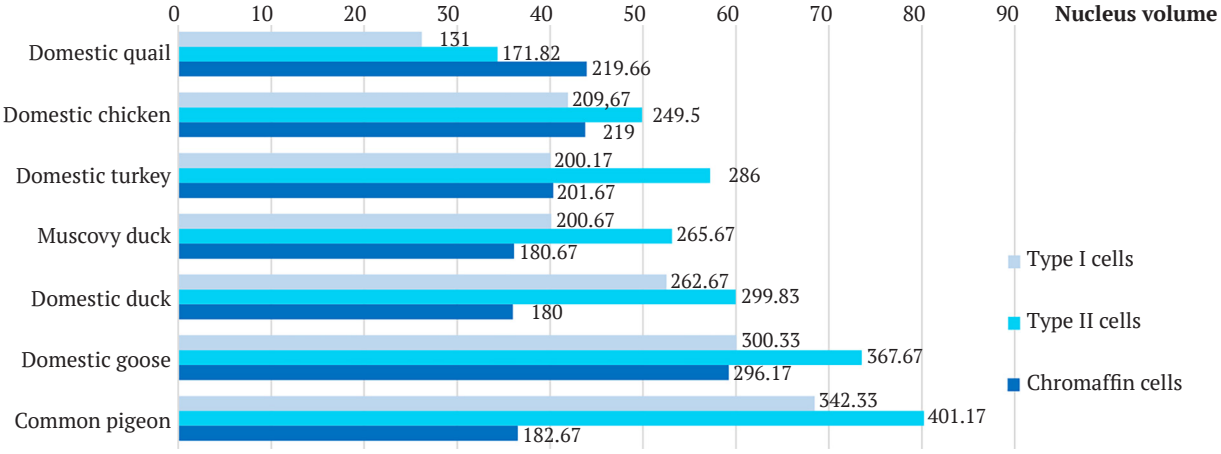

Figure 5. Cytoplasm volume (μm³) of endocrinocytes of the adrenal gland of birds

Consequently, the cells of the second type of interrenal tissue located in the central adrenal zone of the birds under study are the largest, and probably the most functionally active. They possibly correspond to zona fasciculi cells in the adrenal gland of mammals, which are also characterized by large sizes (Kigata & Shibata, 2018; Zakrevska & Tybinka, 2019). Endocrinocytes of the zona fasciculi of the adrenal gland synthesize cortisone, hydrocortisone, and corticosterone. These hormones regulate the metabolism of carbohydrates, proteins, and lipids, suppress inflammatory processes, and stimulate energy metabolism (Scanes, 2015). According to U. Moawad *et al*. (2017), the cytoplasm of acidophilic cells of the domestic chicken adrenal gland contains many mitochondria, ribosomes, lipid droplets, as well as agranular and granular endoplasmic reticulum.

As noted above, basophilic cells form the suprarenal tissue of the adrenal glands. Individual authors (Fathima & Lucy 2014; Moghadam, & Mohammadpour 2017; El-Desoky & El-Zahraa, 2021) call these cells chromaffin cells because of their ability to reduce chromium, silver, and osmium oxides. The cytoplasm of chromaffin cells contains mitochondria with tubular crystals, ribosomes, endoplasmic reticulum, lipid droplets, and secretory granules. Depending on the shape of the secretory granules, they are divided into two types: norepinephrine cells containing secretory granules with an electron-dense nucleus limited by a light border and adrenaline cells containing homogeneous polymorphic electron-dense secretory granules (Moawad & Hassan, 2017). It is proved that hormones of adrenal chromaffin cells and mediators of the sympatho-adrenal system belong to the main regulators of adaptive responses of the body and to exo- and endogenous factors. The biological effects of catecholamines contribute to the transition of the body from a state of rest to a state of arousal, and also ensure its stay in this state for a long time (Scanes, 2015).

In the specialized literature, information on the morphometry of chromaffin cells of the adrenal gland is contradictory and incomplete (Matos, 2008; Kober *et al*., 2012; El-Desoky & El-Zahraa, 2021). According to the results of morphometric studies, it was found that the chromaffin cells of the adrenal glands of birds were characterized by a large nucleus. Its volume exceeded that of cells of the second type of interrenal tissue and was 25.32 ± 1.05 μm³ (domestic quail), 20.83 ± 2.06 μm³ (domestic chicken), 22.17 ± 1.22 μm³ (domestic turkey), 21.83±1.42 μm³ (Muscovy duck), 22.50 ± 1.02 μm³ (domestic duck), 33.50 ± 3.68 μm³ (domestic goose), 26 ± 1.69 μm³ (common pigeon). Therewith, the significant ($P < 0.05$) difference between these indicators was recorded in domestic quail, domestic goose, and common pigeon. In terms of the volume of chromaffin cells, this indicator is probably smaller compared to the volume of cells of the second type of interrenal tissue of the domestic chicken by 1.13 times, $P < 0.01$ (219 ± 24.09 μm³); domestic turkey – by 1.42 times, $P < 0.001$ (201.67 ± 16.59 μm³); Muscovy ducks – by 1.47 times, $P < 0.001$ (180.67 ± 13.16 μm³); domestic ducks – by 1.67 times, $P < 0.001$ (180 ± 20.22 μm³), domestic geese – by 1.24 times, $P < 0.001$ (269.17±26.15 μm³), common pigeon – by 2.2 times (182.67 ± 17.60 μm³). Compared to the volume of cells of the first type, the volume of chromaffin cells decreased by 1.11 times ($P < 0.05$) in Muscovy duck and by 1.46 times ($P < 0.05$) in domestic duck. In domestic quail, on the contrary, the volume of chromaffin cells significantly ($P < 0.001$) exceeded the volume of cells of the first and second type of interrenal tissue by 1.67 and 1.28 times, respectively, and amounted to 219.66 ± 19.33 μm³ (Figs. 4, 5).

The volume of the nucleus and cytoplasm of somatic cells are unstable values. Their average values vary with the age of animals, biological rhythms, gender, etc. (Gyorfy *et al*., 2021). Therefore, during the analysis of the morphological and functional activity of cells, according to the results of cytometric indicators, it is advisable to consider the nuclear-cytoplasmic ratio of endocrinocytes, which, undoubtedly, is a criterion for evaluating the endocrine function of the adrenal gland (Zakrevska & Tybinka, 2019). It was found that domestic quail, chicken, turkey, duck, goose, Muscovy duck, and blue pigeon have the lowest nuclear-cytoplasmic ratio inherent in cells of the second type of interrenal tissue (respectively: 0.092 ± 0.016, 0.082 ± 0.009, 0.083 ± 0.010, 0.072 ± 0.010, 0.066 ± 0.005, 0.076 ± 0.007, and 0.052 ± 0.004), slightly larger – for cells of the first type of interrenal tissue (respectively: 0.109 ± 0.009, 0.097 ± 0.012, 0.111 ± 0.012, 0.081 ± 0.008, 0.079 ± 0.007, 0.110 ± 0.015, and 0.065 ± 0.004), and the largest – for chromaffin cells of the suprarenal tissue (respectively: 0.113 ± 0.014, 0.102 ± 0.015, 0.112 ± 0.010, 0.138 ± 0.007, 0.126 ± 0.006, 0.137 ± 0.003, and 0.166 ± 0.018) (Fig. 6). Such ambiguous cytometric parameters of endocrinocytes may be associated with their various morphofunctional activities, specifically with the development of their hormone-synthesizing apparatus and the state of metabolism.

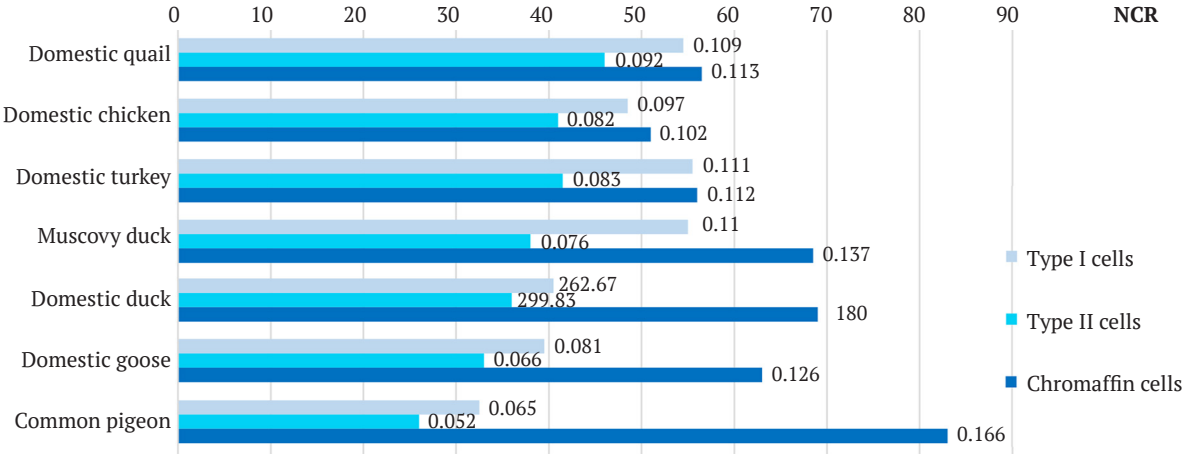

Figure 6. The nuclear-cytoplasmic relationship of endocrinocytes of the adrenal gland of birds

Thus, the adrenal gland of the birds under study has certain differences in morphometric parameters of microstructural components. They can be used to create a base for the normal morphological characteristics of the adrenal gland. This allows assessing the morphofunctional state of the adrenal gland of birds of individual species under a range of factors and pathology.

Conclusions

Morphometric parameters of the microstructural components of the adrenal gland of birds of the Galliformes order (domestic quail, domestic chicken, domestic turkey), Anseriformes (Muscovy duck, domestic duck, domestic goose) and Columbidae (common pigeon) are not the same. They also differ in individual species within the same order. The thickness of the adrenal capsule of birds directly depends on their body weight. Values of indicators of the area of interrenal and suprarenal tissues testify to different morphofunctional activity of these tissues, specifically to more intensive development of interrenal tissue in the peripheral zone of the adrenal gland of all birds under study. In the central zone of the adrenal gland of domestic quail, chicken, and turkey, the indicators of the area of interrenal and suprarenal tissues probably do not differ, which is an inherent morphometric feature of this microstructure of the adrenal gland of chickens.

Indicators of the area of venous sinuses in the central and peripheral zone of the adrenal gland of the common pigeon (Columbidae order) do not differ significantly, which corresponds to the uniform placement of venous sinuses in the parenchyma of the organ under study. In the adrenal gland of birds of the Galliformes and Anseriformes orders, the central zone, in contrast to the peripheral one, is characterized by greater vascularization, which was reflected in the values of the venous sinus area indicator. The nuclear-cytoplasmic ratio is the smallest in the cells of the second type of interrenal tissue (from 0.052 ± 0.004 in common pigeon to 0.092 ± 0.016 in domestic quail), slightly higher in the cells of the first type of interrenal tissue (from 0.065 ± 0.004 in common pigeon to 0.111 ± 0.012 in domestic turkey) and the largest in the chromaffin cells of the suprarenal tissue (from 0.102 ± 0.015 in domestic chicken to 0.166 ± 0.018 in common pigeon), which is associated with the morphological and functional activity of endocrinocytes, namely with the development of their hormone-synthesizing apparatus and the state of metabolism.

Further research intends to involve the histochemical study of the structural components of the adrenal glands in birds at the tissue and cellular levels.

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Мікроморфометрична характеристика надниркової залози птахів

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Анотація. Надниркова залоза – периферичний орган ендокринної системи, який має безпосередній вплив на формування показників продуктивності птахів, що є важливою характеристикою для розвитку промислового птахівництва. Мета роботи полягала у проведенні морфометричної оцінки мікроструктурних компонентів надниркової залози птахів ряду Куроподібні (свійські перепел, курка, індик), Гусеподібні (індокачка, свійські качка і гуска) та Голубоподібні (голуб сизий). Використано анатомічні, гістологічні, морфометричні та статистичні методи досліджень. Встановлено, що товщина капсули надниркової залози птахів прямо залежить від маси їх тіла і варіює від 10,82 ± 0,56 мкм (свійський перепел) до 28,53 ± 1,36 мкм (свійський індик). Інтерреналова тканина порівняно з супрареналовою тканиною в індокачки, голуба сизого, свійських качки і гуски займає більшу ($P < 0,001$) площу центральної зони (відповідно в 3,50, 2,77, 3,10 і 3,11 раза) і периферичної зони (відповідно в 1,27, 2,71, 1,38 і 1,55 раза) надниркової залози, що свідчить про її морфофункціональну активність. Площа венозних синусів у центральній зоні порівняно з периферичною зоною надниркової залози більша у свійського перепела – в 2,80 раза ($P < 0,05$), свійської курки – в 3,62 раза ($P < 0,05$), свійського індика в 3,68 раза ($P < 0,05$), індокачки в 5 разів ($P < 0,01$), свійської качки в 3 рази ($P < 0,05$), свійської гуски в 2 рази ($P < 0,05$). Для голуба сизого характерне рівномірне розміщення венозних синусів по всій периферії надниркової залози і як наслідок близькі за значенням показники їх площі у периферичній та центральній зонах. Показник ядерно-цитоплазматичного відношення ендокриноцитів надниркової залози птахів різниться, зокрема найменший він у клітинах другого типу інтерреналової тканини (від 0,052 ± 0,004 у голуба сизого до 0,092 ± 0,016 у свійського перепела), дещо більший – у клітинах першого типу інтерреналової тканини (від 0,065 ± 0,004 у голуба сизого до 0,111 ± 0,012 у свійського індика) і найбільший – у хромафінних клітинах супрарналової тканини (від 0,102 ± 0,015 у свійської курки до 0,166 ± 0,018 у голуба сизого). Встановлені особливості морфометричних показників мікроструктурних компонентів надниркової залози птахів рекомендовано використовувати для створення бази її нормальної морфологічної характеристики. Це дасть можливість робити оцінку морфо-функціонального стану надниркової залози в умовах впливу різних факторів і за патології

Ключові слова: зони надниркової залози, інтерреналова і супрареналова тканини, венозні синуси, ендокриноцити, ядерно-цитоплазматичне відношення

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Microscopic changes in the spleen due to feline infectious peritonitis

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Abstract. The relevance of the study is that pathological and morphological changes with feline infectious peritonitis have been studied by few authors and are not fully described. The purpose of this study was to investigate the effect of the causative agent of infectious peritonitis on the structure of the spleen in cats. The paper highlights the results of histological studies of sections obtained from distinct parts of the spleen of cats of different ages who died from mixed (26 animals) and dry (7 animals) forms of infectious peritonitis. Sections were stained with haematoxylin and eosin according to the generally accepted method. The paper describes the details of microscopic changes in the spleen in dry and mixed forms of feline infectious peritonitis. It was found that these changes are not affected by the form of the disease but are characterized by features depending on the duration of its course. In cats in which the disease lasted up to three weeks before death, the red pulp of the spleen was unevenly swollen, infiltrated by lymphocytes and monocytes, in some places contained foci of necrotic cells, and red blood cells were absent. Changes in the white pulp were represented by hyperplasia of lymphoid nodules. These nodules were of varied sizes and were located eccentrically relative to the central arteries. There are no distinct lymphoid nodules around part of the central arteries. On the surface of the capsule, fibrinous-necrotic overlays are present in places, under which there is no mesothelium, and the capsule is infiltrated with lymphocytes and monocytes. In other areas, mesotheliocytes underwent distinct metaplasia – from flat cells, they turned into columnar cells. In some areas of the spleen, some animals have no serous membrane. In cats with the disease lasting over three weeks, the red pulp is noticeably more swollen, and the lymphoid nodules are single and small. Other microscopic changes were the same as in animals that were ill for less than three weeks. The results of the study are of practical value for pathologists, as well as for scientists investigating the pathogenesis of feline infectious peritonitis

Keywords: coronavirus, macroscopic changes, histological studies, lymphoid nodules, metaplasia

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Introduction

Coronaviruses in humans and animals cause diseases of the respiratory system, gastrointestinal tract and central nervous system (Enjuanes *et al.*, 2006; Boley *et al.*, 2020). They can adapt to new environments through mutations and recombination with relative ease, and therefore are programmed to effectively change the host range and tissue tropism (Li *et al.*, 2006; Graham & Baric, 2010; Li, 2013). One of the diseases caused by coronaviruses is feline infectious peritonitis, a disease diagnosed in cats worldwide (Andrew, 2000). Currently, feline infectious peritonitis is considered as a viral immunological disease (Kipar *et al.*, 2001).

The coronaviruses affecting this animal species exist in the form of two pathogenic types – low-virulent, called feline intestinal coronaviruses, and virulent – feline infectious peritonitis viruses. The first pathogenic type dominates, since infection with feline coronaviruses occurs by faecal-oral route and pathogens, first of all, enter the intestines. Therewith, the disease does not progress, or manifests itself only with mild enteritis. However, in 4-5% of adult cats and 5-10% of kittens, at some point, after exposure to coronaviruses, infectious peritonitis develops (Addie *et al.*, 2009), as a result of de novo emergence of highly virulent coronaviruses that are formed from low-virulent coronaviruses through mutations in the body of a particular infected cat. This usually occurs without horizontal transmission of the pathogen (Vennema *et al.*, 1998).

In vitro studies have proved that the expansion of the spectrum of cellular pathogenicity of feline coronaviruses, which ensures the growth of their virulence, occurs as a result of efficient and stable replication in macrophages, and not as a result of the expansion of the ability to penetrate the cell (Stoddart *et al.*, 1989).

Presently, it is known that the infectious peritonitis virus is a mutation of the feline intestinal coronavirus, leading to the formation of numerous infectious peritonitis viruses (Stoddart *et al.*, 1989; Vennema *et al.*, 1998). Mutations of enteric coronavirus to infectious peritonitis virus occur frequently and relatively mildly, and probably, occur in a unique and highly variable region of the feline enteric coronavirus genome. At the end of the last century, it was established that infectious peritonitis viruses evolved as deletion mutations of feline intestinal coronaviruses (Poland *et al.*, 1996; Vennema *et al.*, 1998). To date, two genotypes of feline coronavirus are known: feline type I coronavirus, which prevails in the field (Addie *et al.*, 2003; Li *et al.*, 2019) and feline type II coronavirus, which emerged as a result of recombination between feline coronavirus type I and canine coronavirus (Decaro *et al.*, 2008; Terada *et al.*, 2014).

Both serotypes of feline coronavirus can cause infectious peritonitis, but cat populations are certainly dominated by type I coronaviruses, with the number of seropositive animals reaching 98%. Therewith, feline type I coronaviruses induce higher antibody titres than type II coronaviruses and are more likely to cause intestinal diseases and infectious peritonitis. Therewith, a higher prevalence of type II coronaviruses was reported, which ranged from 10% to more than 30% in cats with infectious peritonitis, and sometimes mixed infection with type I and II coronaviruses (Kipar *et al.*, 2014).

According to Levy *et al.* 1999), in 70% of cases, the disease is registered in animals under the age of 1 year. According to Addie & Jarrett (1998), infectious peritonitis affects cats of all ages, but typically 50% of affected cats are under 2 years of age. At the same time, McReynolds & Macy (1997) emphasize that all cats are susceptible to infection with infectious peritonitis, but the clinical manifestation of the disease is most often observed in young cats aged 3 months to 3 years. Furthermore, it was established that this disease is more common in non-neutered cats compared to neutered ones (Pesteanu-Somogyi *et al.*, 2006; Hartmann, 2000). The incidence of infectious peritonitis is not affected by the age of cats, their gender, methods, and methods of raising and keeping, as well as the presence or absence of concomitant diseases (Foley *et al.*, 1998).

Based on pathological and morphological changes, there are three forms of infectious peritonitis: wet, dry, and mixed forms. In case of the latter, changes inherent in both dry and wet infectious peritonitis are recorded. The wet form of the disease is characterized by polyserositis (chest and abdominal effusion) and vasculitis, and the dry form by granulomatous lesions of various organs (Kipar *et al.*, 2005).

In infectious peritonitis, the pathogenesis of the disease and pathological and morphological changes are understudied (Kipar *et al.*, 2014). In this regard, the purpose of this study was to establish pathological and morphological changes in the spleen of cats in mixed and dry forms of infectious peritonitis.

Materials and Methods

The study was conducted during 2019-2022 at the Faculty of Veterinary Medicine of the National University of Life and Environmental Sciences of Ukraine (Kyiv). As a result, a histological examination of the spleen was performed from 26 cadavers of cats that died from a mixed form of infectious peritonitis, 7 cadavers of animals that died from a dry form of this disease, and 5 cadavers of cats (control) that died as a result of lethal injuries. All cats were of different breeds and ages.

The autopsy of the cadavers of all cats was performed by partial evisceration in the dorsal position (Zon *et al.*, 2009). According to this method, after external examination of the cadaver, skin removal and examination of subcutaneous tissue, skeletal muscles and somatic lymph nodes, all organs of the oral, neck, and chest cavities were removed in a single complex, after which they were examined, paying attention to macroscopic changes visible to the naked eye. Organs of the gastrointestinal tract were separated from the abdominal and pelvic cavities as a single complex, and all macroscopic changes visible to the naked eye were recorded. Together with the organs of the gastrointestinal tract, the mesentery, mesenteric lymph nodes, and pancreas were removed and examined. Macroscopic changes in the liver, spleen, kidneys with ureters, adrenal glands, and bladder were removed from each cat's corpse separately, and genitals were removed and investigated in a single complex.

During the autopsy, pieces of the spleen were selected for histological studies. From each spleen, at least 5 pieces were obtained from various parts of the organ – from its central and peripheral parts. In cats that died from infectious peritonitis, areas of the organ were necessarily selected for histological studies, both covered from the surface with fibrinous-necrotic overlays, and without them.

All pieces of spleen were fixed in a 10% aqueous solution of formalin (pH 7.2-7.4) for a week, dehydrated at room temperature in a series of ethanol of increasing concentration (60°, 70°, 80°, 96°, and 100°) and kept in each of them for one day. Subsequently, they were transferred to a mixture of 100° ethanol and chloroform in a 1:1 ratio, where they were kept for one day at room temperature. Next, these pieces were placed in chloroform, in which they were also kept for one day at room temperature, and subsequently, they were kept in a thermostat TSO-80 (Ukraine) in a mixture of chloroform and paraffin in a 1:1 ratio at 37°C. Then, in the same thermostat, they were kept in liquid paraffin at 56°C. After that, pieces of spleen were poured into paraffin. Histological sections were made using a sledge microtome MS-2 (Ukraine), pasted onto glass slides with a mixture of egg albumin and glycerol in a 1:1 ratio, and then the sections were stained with Carazzi haematoxylin and eosin (Goralskij *et al.*, 2011). During staining, the sections were deparaffinized in xylene for 7 min and passed through 100º and 70º ethanol, keeping in each of them for 5 min. Further, the sections were stained with Carazzi's haematoxylin. Subsequently, they were stained with a 0.5% aqueous solution of eosin, dehydrated in 70º and 100º ethanol, clarified in xylene, and embedded in Canadian balsam. The manufactured histological preparations were investigated and photographed using an MC 100 LED microscope (Austria).

Results and Discussion

During the autopsy, it was found that macroscopic changes in the spleen of all cats that died from mixed and dry forms of infectious peritonitis are similar, but have certain features, depending on the duration of the disease. Thus, in animals that were ill for less than 3 weeks before the onset of death, fibrinous-necrotic overlays of varied sizes, shapes, and localization were found on the surface of the organ. Analogous fibrinous-necrotic overlays on the surfaces of various internal organs of cats that died from a dry form of infectious peritonitis were described by other authors (Wolfe & Gricsemer, 1966; Kipar *et al.*, 2005). Compared to animals in control, the pulp of the spleen was somewhat pale.

In animals that were ill for more than 3 weeks before death, apart from fibrinous-necrotic overlays on the surface of the spleen and paler pulp, compared to cats in the control, atrophy of this organ was established. Macroscopically, such atrophy was manifested by a flaccid, sometimes (in 7 animals, or 21.2% of cases) slightly wrinkled capsule and flaccid pulp.

The results of histological studies of the spleen of cats who died from infectious peritonitis show that microscopic changes in this organ were similar in animal corpses, regardless of the form of the disease, age, and gender. However, these changes varied depending on the duration of the disease before death.

In animals that were sick for up to 3 weeks before the onset of death, fibrinous-necrotic overlays were found on the surface of the capsule in places (Fig. 1).

A small number of unchanged lymphocytes and monocytes were found in these fibrinous-necrotic overlays (Fig. 2).

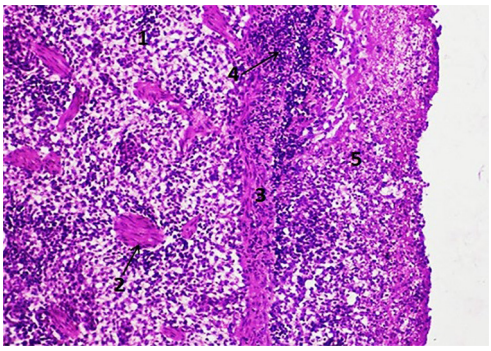

Figure 1. Spleen of a cat with infectious peritonitis for less than 3 weeks. Carazzi's haematoxylin and eosin, x 50
Note: *1 – red pulp; 2 – trabeculae; 3 – capsule; 4 – infiltration of the capsule by lymphocytes and monocytes; 5 – fibrinous-necrotic overlays*

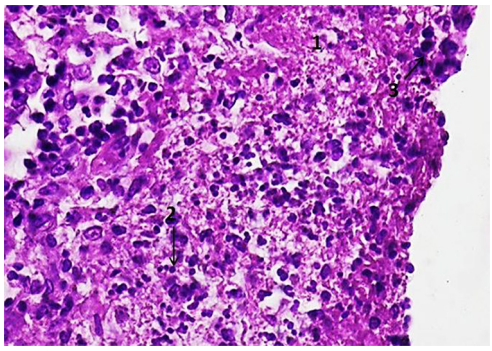

Figure 2. Fibrinous-necrotic overlays on the capsule of the spleen of a cat that had infectious peritonitis for less than 3 weeks. Carazzi's haematoxylin and eosin, x 400
Note: *1 – fibrinous-necrotic masses; 2 – lymphocytes; 3 – monocyte*

Therewith, macrophages were not detected, which indicates the absence of transformation of monocytes into macrophages, which could be explained by both the lack of appropriate stimulation and damage to the mechanisms of the monocytes themselves responsible for such transformation.

Mesothelium under fibrinous-necrotic overlays was absent, and the spleen capsule in these areas was infiltrated by lymphocytes and monocytes (Fig. 1). Other researchers have only stated the presence of such overlays, but have not described their composition, namely intact lymphocytes and monocytes and destruction of mesotheliocytes under such overlays (Kipar & Meli, 2014).

At the same time, it was established that the capsule of the spleen is focally or diffusely infiltrated by lymphocytes and monocytes under fibrinous-necrotic overlays. Focal infiltration in the spleen capsule revealed foci of a fairly dense accumulation of these cells, and diffuse infiltration found single lymphocytes and monocytes located remotely from each other.

In the wet form of infectious peritonitis, the cell infiltrate is represented mainly by neutrophils and macrophages and a small number of T-lymphocytes with plasma cells (Marioni-Henry *et al.*, 2004). However, neutrophil infiltration is inherent in bacterial infections, and therefore

it can be registered in cats with infectious peritonitis complicated by bacterial pathogens. During bacteriological studies, Wolfe & Gricsemer (1966) found out that from patients with feline infectious peritonitis with infiltration of affected tissues and organs by macrophages, neutrophils, lymphocytes and plasma cells, a variety of bacterial microflora is secreted – non-haemolytic staphylococcus, haemolytic streptococcus, enterococci, and E. coli.

Other authors have found that with the dry form of infectious peritonitis, the cellular infiltrate consists of macrophages and B-lymphocytes and impurities of plasma cells (Pedersen, 2009). Microscopic studies found that it is infiltration by monocytes (macrophage precursors) and lymphocytes that occurs in both dry and mixed forms of this disease.

In areas of the organ surface without fibrinous-necrotic overlays, significant microscopic changes were detected in the serous membrane and in the spleen capsule, although these morphological formations stayed unchanged in some areas of the organ. Spleen capsule – swollen (Fig. 3).

Therewith, there is no swelling in the trabeculae in all forms of the disease under study (Fig. 4).

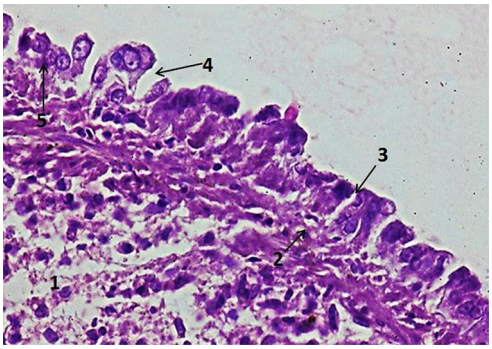

Figure 3. Spleen of a cat with infectious peritonitis for less than 3 weeks. Carazzi's haematoxylin and eosin, x 400
Note: *1 – swelling of the red pulp; 2 – swelling of the connective tissue capsule; 3 – discomplexation of columnar mesotheliocytes; 4 – separation of mesotheliocytes from the spleen capsule; 5 – destruction of mesotheliocytes*

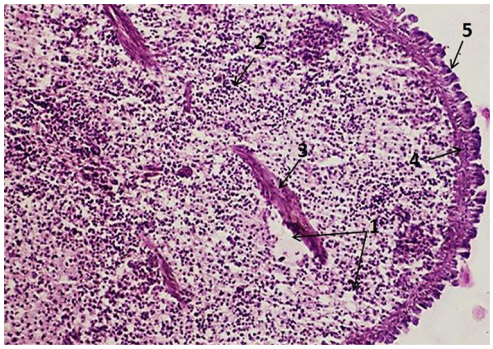

Figure 4. Spleen of a cat with infectious peritonitis for less than 3 weeks. Carazzi's haematoxylin and eosin, x 50
Note: *1 – swelling of the red pulp; 2 – infiltration of red pulp by lymphocytes and monocytes; 3 – trabeculae; 4 – connective tissue capsule; 5 – columnar mesothelium*

Mesotheliocytes of the serous membrane in most areas of the spleen underwent pronounced metaplasia – from flat cells, the long axis of which is oriented parallel to the surface of the capsule of the organ, they turned into cubic and columnar cells (Fig. 3).

The authors of this study believe that this is a reflection of the stages of such metaplasia: initially, due to a gradual increase in the volume of the cytoplasm, cubic-shaped cells are formed, and with a subsequent increase in the volume of the cytoplasm, these cells acquire a columnar shape.

The nuclei of mesotheliocytes that underwent metaplasia noticeably increased in size. They, both in cubic and columnar cells, acquired a rounded or somewhat oval shape, contained one, rarely two nucleoli. A significant part of such nuclei was occupied by heterochromatin, which, according to modern concepts (Thayer *et al.*, 2022), reflects an increase in the number of active sites of transcription. Such activation of the mesotheliocyte genome indicates an increase in their functional or synthetic activity. Arguably, there is an intensification of the processes of synthesis of cytoplasmic components, as a result of which a noticeable increase in cell volume is observed. Some altered mesothelial cells were destroyed (Figs. 3, 5).

In the spleen of 7 cats, small foci of complete destruction of the organ capsule were recorded on the part not covered with mesothelium (Fig. 6).

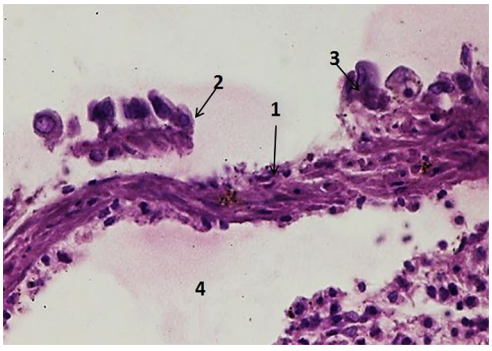

Figure 5. Spleen of a cat with infectious peritonitis for less than 3 weeks. Carazzi's haematoxylin and eosin, x 400
Note: *1 – capsule not covered by mesothelium; 2 – a layer of mesotheliocytes near the outer surface of the capsule; 3 – destruction of mesothelium cells on the surface of the capsule; 4 – subcapsular swelling*

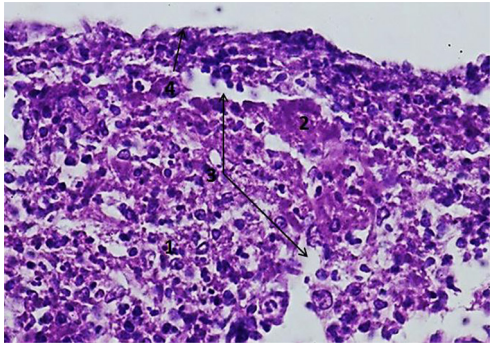

Figure 6. Spleen of a cat with infectious peritonitis for less than 3 weeks. Carazzi's haematoxylin and eosin, x 400
Note: *Spleen of a cat with infectious peritonitis for less than 3 weeks. Carazzi's haematoxylin and eosin, x 400*

Dyscomplexation of the metaplastic cells of the mesothelium and separation of individual mesotheliocytes or their small groups from the spleen capsule was often found (Fig. 3). Such changes indicate considerable violations of cellular contacts. In some places, entire layers of mesotheliocytes were separated from the capsule (Fig. 5). This can be caused by both destruction of intercellular contacts and damage to cell adhesion molecules.

In the red pulp of the spleen, irregularly located foci of marked oedema were found. No red blood cells were observed in the red pulp. Instead, uneven infiltration by lymphocytes and monocytes and foci of necrotic cells were observed (Figs. 1, 3-6). It was not possible to identify which cells of the red pulp of the spleen were necrotic.

Apart from clear changes in the red pulp, hyperplasia of the lymphoid nodules of the spleen was found. Therewith, lymphoid nodules in the spleen of cats that had infectious peritonitis for less than 3 weeks had varied sizes and were located eccentrically relative to the central arteries. These arteries were localized not in the central part of the lymphoid nodules, which is characteristic of control cats, but in the peripheral areas of these lymphoid formations.

Notably, the red pulp of the spleen around the lymphoid nodules is infiltrated by a noticeably larger number of lymphocytes and monocytes compared to its more distant areas. Therewith, there were no distinct lymphoid nodules around individual central arteries (Figs. 7, 8).

Necrosis and destruction of the walls were found in the central arteries without distinct lymphoid nodules (Fig. 9).

Perhaps such damage to the walls of these arteries led to the fact that lymphoid nodules did not form near them. On the other hand, such changes could be caused by the action of some factor(s) that caused both the absence of lymphoid nodules and damage to the walls of the central arteries.

In cats in which the disease lasted for more than 3 weeks and less than 3 weeks before death, the microscopic changes in the spleen were similar, but they had some features. The red pulp was noticeably more swollen, and the lymphoid nodules were few and small or were not detected at all (Fig. 10).

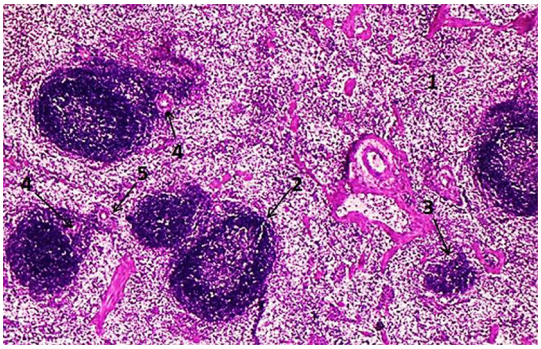

Figure 7. Spleen of a cat with infectious peritonitis for less than 3 weeks. Carazzi's haematoxylin and eosin, x 50
Note: *1 – red pulp; 2 – a large lymphoid nodule; 3 – a small lymphoid nodule; 4 – the central artery in the peripheral part of the lymphoid nodule; 5 – central artery without a distinct lymphoid nodule*

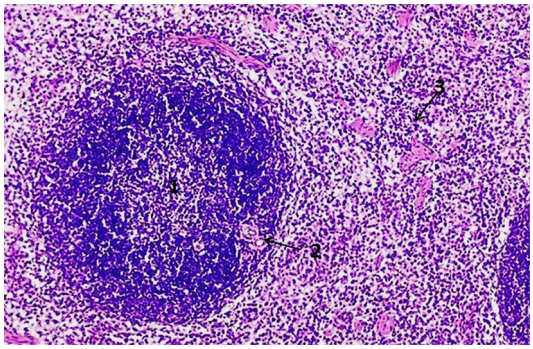

Figure 8. Spleen of a cat with infectious peritonitis for less than 3 weeks. Carazzi's haematoxylin and eosin, x 100
Note: *1 – a large lymphoid nodule; 2 – the central artery in the peripheral part of the lymphoid nodule; 3 – infiltration of the red pulp by lymphocytes and monocytes*

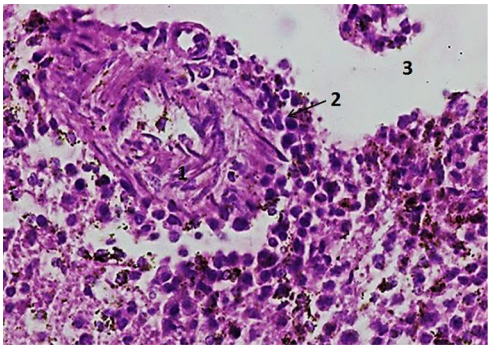

Figure 9. Spleen of a cat with infectious peritonitis for less than 3 weeks. Carazzi's haematoxylin and eosin, x 400
Note: *1 – necrosis and destruction of the wall of the central artery; 2 – a small number of lymphocytes and monocytes near the central artery; 3 – oedema*

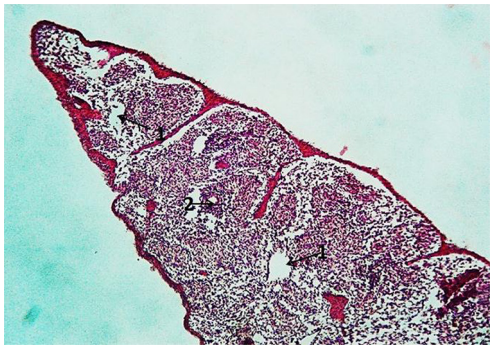

Figure 10. Spleen of a cat with infectious peritonitis for more than 3 weeks. Carazzi's haematoxylin and eosin, x 30
Note: *1 – pronounced swelling of the red pulp; 2 – a small lymphoid nodule with an enlarged central artery*

The described microscopic changes suggest that a long course of infectious peritonitis leads to considerable depletion of the immune system in sick cats. Comparable microscopic changes were observed in animals that had infectious peritonitis for less than 3 weeks.

In general, detailed histological studies of the spleen of cats with infectious peritonitis have not previously been conducted abroad (Thayer *et al.*, 2022). In Ukraine, microscopic changes in the spleen of cats in wet and dry forms of infectious peritonitis are described only in one scientific

paper by Halania (2020). The author found that in case of the wet form of the disease, there is a reduction in white pulp. Analogous changes in the white pulp according to the results of pre-microscopic studies were established in cats in which the disease lasted more than 3 weeks. In the same paper, it is indicated that with the dry form of infectious peritonitis, there is intense blood filling of the red pulp and hyperplasia of lymphoid nodules due to T-lymphocytes.

In the red pulp of the spleen of cats whose disease lasted up to 3 weeks before death, the absence of red blood cells, foci of oedema of assorted sizes, uneven infiltration by lymphocytes, monocytes, and small foci of cell necrosis were found, and in the white pulp – hyperplasia of lymphoid nodules.

Since Halania (2020) did not consider the duration of cat disease before death, the author may have studied animals that had a wet form of infectious peritonitis for more than 3 weeks before death, and a dry form for up to 3 weeks.

Various changes in the red pulp in the dry form of the disease could be caused by changes in some properties of the causative agent of the disease, which is characterized by numerous and rapidly emerging mutations (Poland *et al*., 1996; Vennema *et al*., 1998).

Thus, the conducted studies indicate that with infectious peritonitis, microscopic changes in the spleen of cats depend on the duration of the course of the disease until death. In the initial stages of the disease, the disappearance of red blood cells from the red pulp, mesotheliocyte metaplasia, and signs of activation of the immune system in the form of hyperplasia of lymphoid nodules are recorded. With the further development of the disease, changes in the red pulp and mesothelium persist, and the lymphoid nodules atrophy.

Conclusions

Microscopic changes in the spleen of cats with infectious peritonitis did not depend on the form of the disease, but slightly differed depending on the duration of its course.

In cats in which the disease lasted up to 3 weeks before death, the red pulp of the spleen showed the absence of red blood cells, foci of oedema, uneven infiltration by lymphocytes and monocytes, and foci of necrotic cells.

The number of lymphoid nodules of the spleen increased, and necrosis and destruction of their walls were recorded in part of the central arteries.

In the serous membrane of the spleen, the absence of mesothelium under foci of fibrinous-necrotic overlays and metaplasia of mesothelial cells in other areas was established. The nature of mesotheliocyte metaplasia on the surface of the same spleen is different. In some areas of the organ, mesotheliocytes turn from flat cells into cubic cells, and in other areas they are columnar cells. Such metaplasia in the spleen is established in all cats with various forms of the disease.

In cats in which the disease lasted more than 3 weeks, the red pulp is noticeably more swollen, and the lymphoid nodules are few and small, which indicates the exhaustion of the potential of the immune system.

The prospects of the research involve further detailed investigation of pathological and morphological changes upon feline infectious peritonitis in other organs and tissues.

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Мікроскопічні зміни в селезінці за інфекційного перитоніту котів

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Анотація. Актуальність дослідження зумовлена тим, що патоморфологічні зміни при інфекційному перитоніті у котів вивчалися небагатьма авторами і описані недостатньо повно. Мета роботи полягала у вивченні впливу збудника інфекційного перитоніту на будову селезінки в котів. У роботі висвітлено результати гістологічних досліджень зрізів, одержаних із різних ділянок селезінки котів різного віку, що загинули від змішаної (26 тварин) і сухої (7 тварин) форм інфекційного перитоніту. Зрізи фарбували гематоксиліном та еозином за загальноприйнятою методикою. У роботі детально описано мікроскопічні зміни в селезінці за сухої і змішаної форм інфекційного перитоніту котів. З'ясовано, що на ці зміни не впливає форма хвороби, але відзначаються особливостями залежно від тривалості її перебігу. У котів, в яких хвороба до настання смерті тривала до трьох тижнів, червона пульпа селезінки нерівномірно набрякла, інфільтрована лімфоцитами й моноцитами, подекуди містила вогнища некротизованих клітин, а еритроцити відсутні. Зміни білої пульпи представлені гіперплазією лімфоїдних вузликів. Ці вузлики мали різні розміри та були розташовані ексцентрично відносно центральних артерій. Навколо частини центральних артерій виразні лімфоїдні вузлики відсутні. На поверхні капсули місцями наявні фібринозно-некротичні накладання, під якими мезотелій відсутній, а капсула інфільтрована лімфоцитами та моноцитами. На інших ділянках мезотеліоцити зазнали виразної метаплазії – з плоских клітин вони перетворилися на стовпчасті клітини. На окремих ділянках селезінки у деяких тварин серозна оболонка відсутня. В котів, у яких хвороба тривала більше трьох тижнів, червона пульпа помітно більш набрякла, а лімфоїдні вузлики – поодинокі та невеликих розмірів. Інші мікроскопічні зміни були такими ж, як і у тварин, що хворіли менше трьох тижнів. Результати дослідження мають практичну цінність для лікарів-патоморфологів, а також для науковців, які вивчають патогенез інфекційного перитоніту котів

Ключові слова: коронавірус, макроскопічні зміни, гістологічні дослідження, лімфоїдні вузлики, метаплазія

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Effect of allogeneic blood transfusion on neutrophil functional activity and lymphocyte cytotoxicity in recipient rabbits

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Abstract. The relevance of this paper is that transfusion of allogeneic blood to recipient animals is always associated with immunological risks. In this regard, the purpose of this study was to assess the state of phagocytic activity of blood neutrophils by indicators of phagocytic index, phagocytic number, and oxygen-dependent bactericidal activity, as well as to establish changes in antibody-dependent cytotoxic activity of lymphocytes in recipient rabbits during allogeneic whole blood transfusion. Modelling of blood transfusions was performed on five clinically healthy rabbits by intravenous administration of whole blood at the rate of 5.5 ml/kg of body weight. Blood samples were taken from animals on Days 3, 7, and 23 after blood transfusion. Neutrophil populations were obtained from blood samples by centrifugation on a double density gradient of 1.077 and 1.093 Ficoll-Verografin. The absorption activity of phagocytes was determined in a microscopic test. To investigate the oxygen-dependent bactericidal activity of neutrophils, a spontaneous test with nitroblue tetrazolium was performed. Antibody-dependent cytotoxic activity of lymphocytes was investigated by colorimetric method. It was found that after the transfusion of whole blood, the phagocytic activity of neutrophils increases with a simultaneous decrease in their absorption capacity. On Days 3 and 7, the results of the spontaneous test with nitroblue tetrazolium decreased. This indicates inactivation of the oxygen-dependent bactericidal activity of neutrophil granulocytes during the first phase of post-transfusion immunological reactions. On Day 23, there was an increase in the values of the indicators of the spontaneous test with nitroblue tetrazolium, which indicates the activation of the bactericidal properties of phagocytes. It was found that on Day 3, the antibody-dependent cytotoxic activity of lymphocytes significantly decreased relative to the initial state, and on Days 7 and 23, it increased. An increase in the antibody-dependent cytotoxic activity of lymphocytes should be associated with the active synthesis of antibodies of the late phase of the immune response. Consequently, transfusion of allogeneic blood causes an immune response in recipient rabbits, without causing immediate and long-term transfusion reactions (changes in heart rate, respiratory rate, body temperature). The obtained results are of practical value both for scientists and practising doctors who use transfusion of whole blood and its components to animals with acute anaemia, impaired functional activity of blood coagulation factors, parasitic, and oncological diseases

Keywords: white blood cells, phagocytic index, NBT test, antibodies, granulocytes, immunity

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Introduction

Over the past decade, interest in veterinary transfusiology has increased rapidly. Transfusion of whole blood and its components is vital for parasitic diseases, violation of blood circulation factors, cancer, and polytrauma. That is why whole blood prepared in advance is crucial during intensive veterinary care to sick animals (Lo *et al*., 2019; Yehorov *et al*., 2020).

Reactions to blood transfusions in recipient animals are classified as immunological and non-immunological, as well as acute and delayed (Stepura *et al*., 2020). It is known that acute transfusion reactions are observed in recipient animals (3-8%) after whole blood transfusion (Stepura *et al*., 2020). In such animals, as in humans, whole blood transfusions can cause severe acute respiratory distress syndrome (Irani *et al*., 2017; Brugué *et al*., 2018; Yehorov *et al*., 2021). This condition is characterized by non-cardiogenic pulmonary oedema. It is arguably related to the response of donor antibodies to recipient leukocytes (Yehorov *et al*., 2022). It is also known that blood transfusion in dogs causes a simultaneous increase in the number of neutrophil granulocytes and the content of C-reactive protein, which indicates the development of an inflammatory process in the animal's body (Day *et al*., 2020; Claus *et al*., 2022).

Blood transfusion is considered an allogeneic tissue transplantation operation, which can have serious complications. Despite strict compliance with the requirements concerning the blood group of the donor and the recipient, as well as conducting an *in vitro* compatibility reaction, an immune conflict may occur in the body of the recipient animal. This is because, apart from transplanted erythrocytes, their body receives many immunogenic corpuscular (leukocyte, platelet) and humoral (plasmatic) antigenic factors that can cause both humoral (activation of class M, G immunoglobulins) and cellular immune response. Notably, a non-cardiogenic pulmonary reaction in recipient animals occurs as a result of transfusion of leukocyte antibodies (Ab). Naturally, a pronounced immune conflict in the body of the recipient animal can occur even with a minimal volume of blood transfusion (Yagi & Holowaychuk, 2016; Poh *et al*., 2018).

Taylor *et al*. (2021) argue that transfusion responses in cats are unpredictable and vary in severity. Post-transfusion reactions can be both acute and chronic. The most common transfusion reactions are manifested in the form of fever, allergies, as well as circulatory overload associated with transfusion. Transfusion reactions can trigger immune-mediated hemolysis, which causes jaundice and an increase in body temperature, which occurs due to the development of an antigen-antibody complex. Such reactions can also lead to non-hemolytic consequences – fever, itching of the skin, swelling in the facial area of the head (Taylor *et al*., 2021).

It is known that phagocytes are essential in maintaining the constancy of the immune status of the animal body. As non-specific protection factors, they not only capture and digest microorganisms, but also activate specific mechanisms of resistance of the macroorganism. Therefore, it is important to investigate the functional state of phagocytes, specifically neutrophils (Shcherba & Korda, 2018).

The leading characteristic of granulocytes is the assessment of their phagocytic activity. Its decrease can be the result of both a lack of opsonizing factors in the serum, and defects in the cells themselves. It is known that white blood cells can change their morphological and functional state, taking part in the immune processes of the mammalian body (Yelyseyeva *et al*., 2020). This ability acts as a criterion for the degree of activity and the nature of the process. Among white blood cells, neutrophils, due to their functional activity, play an important role in the humoral-cellular system of cooperation in the immune response to the antigen. That is why in the mammalian body, neutrophils are both indicators and a universal target for various disorders of homeostasis. Their quantitative and qualitative composition ensures leukocyte activity and is manifested primarily by phagocytic activity (Yelyseyeva *et al*., 2020).

The hereditary immune system includes large granular lymphocytes – NK cells. Under interleukin-15 (IL-15), they are formed from a lymphocyte stem cell in the bone marrow (Lo *et al*., 2019). They belong to the system of innate immunity since they develop without rearrangement of the genes of the receptor apparatus. However, unlike other innate immune cells, they recognize molecular structures of antigenic origin that are foreign to the mammalian body. NK cells detect and neutralize altered cells in their own body. At the same time, their targets are cells of the own body that have partially or completely lost the expression of major histocompatibility complex (MHC) class I molecules but express a normal or increased level of surface molecules induced by cellular stress (Lo *et al*., 2019; Baeva, 2019; Abbas *et al*., 2020).

Such molecular changes are inherent in cells infected with viruses, cytosolic parasites, as well as for tumour cells. NK cells can neutralize target cells without any prior activation (immunization). This property is called natural cytotoxicity (NK activity) and underlies the term "natural killers". Furthermore, due to the expression of Fc receptors, NK cells can take part in antibody-dependent cellular cytotoxicity reactions, i.e., neutralize IgG-opsonized antigens. Involvement of Fc receptors in the recognition of antigen epitopes considerably increases the effectiveness of cytolysis (Baeva, 2019).

Neutralization (lysis) of infected macroorganism cells and tumour cells is the main function of NK cells. Due to the ability to independently recognize "own-foreign" on the surface of the target cell, cytolysis by NK cells occurs, which distinguishes them from cytotoxic T-lymphocytes. Lysis of target cells is provided by perforin and granzymes – proteins found in the lytic granules of NK cells. During their interaction with target cells, lysis of the latter occurs due to perforins and granzymes – proteins stored in the lytic granules of NK cells, while during their interaction with the target cell, degranulation occurs. Granzymes, entering the cytosol of the target cell with perforin, induce its apoptosis (Lo *et al*., 2019; Kyivska, 2019).

It is known that phagocytes play an important role in protecting the tissues of the mammalian body and supporting the constancy of the immune status of the body. These cells act as non-specific defence factors that not only phagocytize and process the antigen (Ag), but also mobilize specific resistance mechanisms of the animal body (Legenchuk & Nemyrovych, 2022). Therefore, it is important to understand allogeneic tissue transplantation to investigate the functional state of phagocytes by oxygen-dependent bactericidal activity of blood neutrophils using the nitroblue tetrazolium test (NBT test) (Stasenko, 2019).

The purpose of this study was to investigate the functional properties of neutrophilic granulocytes and antibody-dependent cytotoxic activity of lymphocytes in recipient animals by allogeneic blood transfusion.

Materials and Methods

The study was conducted during 2021-2022 at the Scientific and Educational Laboratory "Animal Blood Bank" of the Acad. I.O. Povazhenko Department of Surgery and Pathophysiology and in the conditions of the Educational and Scientific Production Clinical Centre "Vetmedservice" of the National University of Life and Environmental Sciences of Ukraine.

Experiments on rabbits were conducted per the requirements of the "General Ethical Principles of Conducting Experiments on Animals", approved by the First National Congress on Bioethics (September 20, 2004, Kyiv, Ukraine) (Law of Ukraine..., 2006), the Regulations of the "European Convention on the Protection of Vertebrates Used for Experimental and Other Scientific Purposes" (Strasbourg, 1986) (European Convention for..., 1986) and the Law of Ukraine "On the Protection of Animals from Cruel Treatment" (2006).

Five clinically healthy rabbits of the White Pannon breed were used in the experiments. The diet of experimental animals corresponded to their need for nutrients and biologically active substances. The animals had free access to water and food.

Blood from donor animals was taken from the jugular vein by a semi-closed method. At the blood sampling site, the animals' fur was shaved, and the skin was treated with a 70% alcohol solution. During the selection of animal blood, the tissues in the place where the jugular vein passes were moderately stretched. The head of the animal was diverted to the side opposite to the side of blood sampling. A jugular vein puncture was performed in the upper and middle third of the neck. At an angle of 45°, a needle was inserted, piercing the skin and vein wall against the blood flow. For the convenience of manipulation, the vein below the puncture site was pressed with the finger of the left hand. Donor blood samples of rabbits were collected in polymer containers with the CPDA anticoagulant (citrate, phosphate, dextrose, and adenine). Recipient rabbits received whole blood transfusions in the amount of 5.5 ml/kg of body weight.

The study was based on blood plasma samples taken from five rabbits on Days 3, 8, and 23 after transfusion. The population of blood neutrophils was obtained by centrifugation (Hettich EVA laboratory centrifuge, Germany) on a double density gradient of 1.077 and 1.093 Ficoll-Verografin (Yamashita *et al.*, 2019).

The phagocytic activity of blood granulocytes (absorbing activity of phagocytes) was determined in a microscopic test. When investigating the phagocytic activity of neutrophils, standard latex particles for phagocytosis (10% polystyrene suspension) with a diameter of 1.0-1.3 μm were used as test systems. The functional activity of phagocytes was assessed by determining phagocytic number (PN), % is the number of phagocytizing cells per 100 counted; phagocytic index (FI), c.u. is the average number of latex captured by one phagocyte.

A suspension of neutrophil cells was introduced into a test tube in the amount of 0.1 ml and incubated at 37°C for 60 min with 0.1 ml of latex suspension. Smears were prepared on glass slides, dried, fixed, and stained according to Romanowsky-Giemsa. 100 neutrophils were counted in each smear (Crawford *et al.*, 2022).

The oxygen-dependent bactericidal activity of blood neutrophils was determined by a spontaneous nitroblue tetrazolium test (HBT test). When setting up a spontaneous HBT test, phagocytes were cultured in the presence of nitroblue tetrazolium without prior cell activation (Grasso *et al.*, 2019). The oxygen-dependent bactericidal activity of neutrophils in the HBT test was investigated using the spectrophotometric method (Grasso *et al.*, 2019). A suspension of phagocytes in culture medium 199 (1×106 cells/ml) was introduced into centrifuge tubes and centrifuged at 1,000 rpm for 5 minutes. Subsequently, the supernatant fraction was removed, and 0.2 ml of HBT solution in a phosphate-salt buffer with a mass fraction of 0.2% was added to the phagocytes. Cells were cultured for 60 min at 37°C in a water-saturated atmosphere with a constant CO_2 level (5%). After incubation, the tubes were centrifuged at 1,000 rpm for 1-2 minutes. Then the supernatant fluid was removed, and 0.2 ml of HCl was added to the phagocytes. The test tubes were thoroughly shaken and centrifuged again under the same conditions. Subsequently, the supernatant fraction was removed from the test tubes and 0.2 ml of dimethyl sulphoxide was added for phagocyte lysis. After cell lysis, 3 ml of phosphate-salt buffer was added to the test tubes. The optical density of diformazan in the samples was measured on a Ulab 102 UV spectrophotometer (China) at an optical wavelength of 492 nm in cuvettes (1×1 cm) against a solution of 2.8 ml of phosphate buffer with 0.2 ml of dimethyl sulfoxide (Grasso *et al.*, 2019).

Antibody-dependent cytotoxic activity of lymphocytes was determined by the colorimetric method (Grasso *et al.*, 2019).

Statistical processing of the research results was performed using the "Statistica 5.0" software (StatSoft Inc., USA). Differences between two indicators of the compared samples were considered statistically significant at $P<0.05$, $P<0.01$, $P<0.001$ (Filimonova *et al.*, 2005).

During the studies, the main clinical indicators (body temperature, heart rate, respiratory rate) were monitored in experimental animals.

Results and Discussion

Changes in the phagocytic activity of neutrophil granulocytes in the blood of recipient rabbits during allogeneic whole blood transfusion are presented in Table 1.

Table 1. Dynamics of changes in the phagocytic activity of neutrophilic granulocytes in rabbit blood during allogeneic whole blood transfusion (M ± m, n = 5)

Seq. No.	Cells	Initial state	Day 3	Day 7	Day 23
1	Phagocytic index, c.u.	26 ± 2.50	30 ± 1.50	37 ± 2.80**	39 ± 3.40**
2	Phagocytic number, %	7.86 ± 0.18	7.12 ± 0.13**	6.20 ± 0.18***	6.87 ± 0.24**

Note: ** P<0.05; **P<0.01; ***P<0.001, significant relative to the initial state*

As a result of determining the phagocytic activity of neutrophil granulocytes in rabbits after whole blood transfusion, the phenomenon of "phagocytosis dysfunction" was identified. Thus, during whole blood transfusion, the phagocytic index increased in recipient rabbits on Days 7 and 23 of the experiment by 42.3% and 50.0% ($P < 0.01$), respectively (Table 1).

Therewith, the phagocytic number significantly decreased relative to the initial state on Day 3, Day 7, and Day 23 of the experiment by 9.4% ($P<0.01$), 22.0% ($P < 0.001$), and 13.0% ($P < 0.01$), respectively.

Thus, in case of whole blood transfusion in the body of recipient rabbits, the absorption capacity of blood neutrophils decreases against the background of an increase in their phagocytic activity, which indicates a dysfunction of phagocytosis. The obtained data indicate considerable compensatory capabilities of neutrophils, which can maintain the overall balance in the phagocytic system under conditions of intense load on specific and non-specific links of immunity. Notably, under these conditions, the body temperature of the experimental animals was within the normal range. The established regularities coincide with the results of studies described by other authors (Callan *et al*., 2013; Suddock & Crookston, 2022).

Oxygen-dependent phagocyte metabolism was determined using the NBT test. This test reflects the degree of functional irritation of phagocytic cells and is an indirect indicator of the functional activity of cells. The NBT test was used to characterize the ability of phagocytes to destroy microbial cells, i.e., their antibacterial activity (Fig. 1).

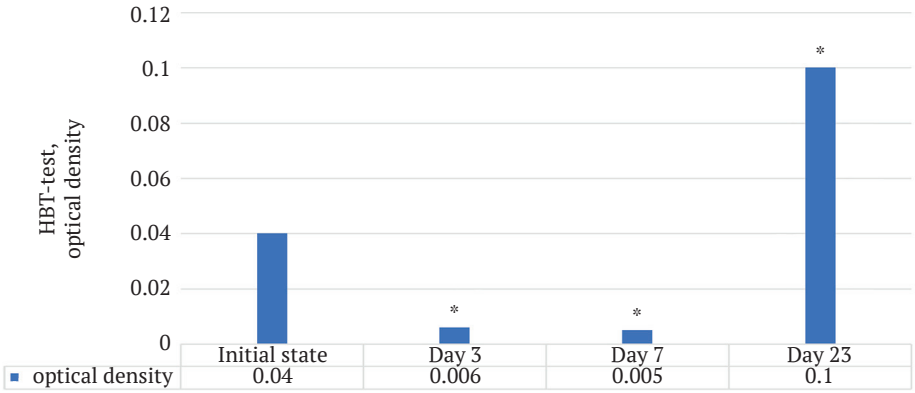

Figure 1. Dynamics of functional activity of neutrophils in the reaction of reduction of nitroblue tetrazolium in rabbit blood during allogeneic whole blood transfusion

Note: **P < 0.05, values are significant relative to the initial state*

Indicator of the NBT test (Fig. 1) in rabbits, whole blood transfusions experienced a decrease of 15% ($P < 0.05$) and 12.5% ($P < 0.05$), respectively, compared to the initial state. At the same time, on Day 23 of the experiment, the level of this indicator increased by 16.6% ($P < 0.05$) relative to the initial state. The established increase in the value of this indicator during whole blood transfusion correlates with the dynamics of the phagocytic index (PI), which was also marked by an increase, but already by 50.0% ($P<0.01$) relative to the initial level (Table 1). Activation of antibacterial oxygen-dependent systems within phagocyte cells on Day 23 after whole blood transfusion probably occurs due to the destruction of transplanted cells in the recipient animal body during this period (Yagi & Holowaychuk, 2016; Day *et al*., 2020).

Portnov (2021) notes that the HBT test scores may also increase at the initial stage of acute bacterial infections. While in the subacute and chronic course of the infectious process, their decrease is noted. Rehabilitation of the body from pathogens is accompanied by the restoration of the values of this indicator. A sharp decrease in its level indicates decompensation against infectious defences and is considered a prognostically unfavourable sign.

A complex of reactions aimed at recognizing, absorbing, and eliminating microscopic particles from the body is called phagocytosis. Kling *et al*. (2020) indicate that many cell types are more or less capable of phagocytosis, but the phagocytic activity of neutrophils is most important for the immune system.

Krüger (1995) noted that the storage of blood affects all its components. Preliminary removal of polymorphonuclear neutrophils from the blood improves the quality of its components. Therewith, a decrease in their number can lead to the disappearance of important mechanisms of antibacterial protection.

Antibody-dependent cytotoxic activity of lymphocytes in the blood of recipient rabbits during allogeneic whole blood transfusion was investigated. The dynamics of antibody-dependent cytotoxic activity of lymphocytes in the blood of recipient rabbits during whole blood transfusion is presented in Figure 2.

On Day 3 of experimental studies, in the case of allogeneic whole blood transfusion to recipient rabbits, the antibody-dependent cytotoxicity of leukocytes significantly decreased by 16% relative to the initial state ($P < 0.01$) (Fig. 2). The described pattern is similar to the results of other researchers (Yagi & Holowaychuk, 2016; Day *et al*., 2020). It is probable that the decrease in the antibody-dependent cytotoxic activity of lymphocytes in the peripheral blood of recipient rabbits on Day 3 of experimental studies relative to the initial state is due to the activation of the activity of the immune system as a whole and the synthesis of class M immunoglobulins (pentamers), to which natural killer cells (NK cells) have no specific receptors. Notably, it is Class M antibodies that are synthesized by plasma cells during the acute period of the immune response, i.e., in the case of first contact with certain epitopes of antigens (Keir *et al*., 2012).

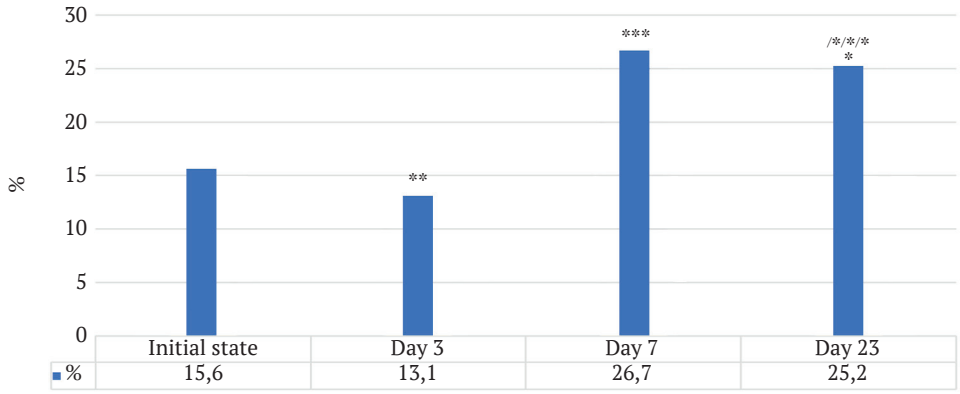

Figure 1. Dynamics of antibody-dependent cytotoxic activity of lymphocytes in the blood of recipient rabbits during allogeneic whole blood transfusion

Note: ** P < 0.05; ** P < 0.01; ***P < 0.001, values are significant relative to the initial state, and /*** P < 0.001, significant compared to the previous stage of the experiment*

On Day 7 of experimental studies (Fig. 2) when whole blood was transfused to recipient animals, the antibody-dependent cytotoxic activity of blood lymphocytes increased by 71.3% (P<0.001) relative to the initial state. Notably, at this time of the experiment, antibody-dependent cellular cytotoxicity increased by 2 times compared to 3 days of the experiment. An increase in antibody-dependent cell cytotoxicity on Day 7 of the experiment relative to the initial state and 3 of its days should be associated with the active synthesis of late-phase immune response antibodies (IgG) that interact with Fc domains with surface receptors of NK cells. Class G immunoglobulins have only 2 antigen binding centres and a lower molecular weight, thereby providing greater specificity with epitopes of corpuscular and humoral antigens (red blood cells, white blood cells, platelets, plasma proteins), thus providing stable avidity (antibody-antigen relationship) (Abbas *et al*., 2020).

On Day 23 of the experiment, the cytotoxic activity of blood lymphocytes significantly increased relative to the initial state by 61.5% (P<0.05), and relative to Day 3 of the experiment by 1.9 times (/*** P<0.001). The increase in antibody-dependent cell cytotoxicity on Day 23 of the experiment on allogeneic whole blood transfusion in recipient rabbits is probably associated with the active synthesis of Class G immunoglobulins by plasma cells, which actively opsonize transplanted antigens, forming an Ag-Ab complex, and thereby activate antibody-dependent cell cytotoxicity reactions (Australian Red Cross..., 2020). At the same time, antibody-dependent cell cytotoxicity tended to decrease by 5.6% compared to Day 7 of the experiment (26.7%). Such changes occur due to apoptosis of transplanted platelets and leukocytes on Days 7-9 days and 14-16 after transfusion, which coincides with the results of research by Day *et al*. (2020).

The decrease in antibody-dependent cellular cytotoxicity in experimental animals is caused by a decrease in the antigenic load, especially corpuscular antigens, on the immune system of recipient animals on Day 23 relative to Day 7 of the experiment. The results obtained are consistent with those of Dani *et al*. (2017), Poh (2018) and Yagi & Holowaychuk (2016).

As noted by Ueta *et al*. (2018), when recipient lymphocytes and donor stimulating cells are co-cultured, cytotoxic T lymphocytes appear among the cells. They can destroy target cells that carry antigens that are also present on stimulating cells. The study of cellular cytotoxicity in mixed lymphocyte culture in some cases allows predicting whether the graft will stimulate the formation of cytotoxic T-lymphocytes or not.

Crawford *et al*. (2019) demonstrated that transfusion of donor-specific blood induces alloresponses and leads to immunosuppression. Using fully allogeneic combinations of rat blood cells in scientific experiments, scientists have found that red blood cell products can directly modulate the function of immune cells. This is called transfusion-associated immunomodulation (TRIM), which provides an appropriate adaptive mechanism. A single blood transfusion effectively induces a T-cell-dependent helper response of antibody-forming cells of the main histocompatibility complex (MHC) Class I with a change in the class of immunoglobulins and a donor-specific response of regulatory lymphocyte cells (Treg). This occurs mainly in the spleen, probably by indirect allorecognition via resident dendritic cells (DCs).

Notably, during the studies in experimental animals, the main clinical indicators (body temperature, heart rate, respiratory rate) stayed within the physiological parameters and corresponded to the range of values of 38.5-39.5°C, 180-250 heartbeats/min, 50-60 respiratory movements/min, respectively.

Thus, the results of the conducted studies indicate that whole blood transfusion causes complex immunological changes in the body of recipient animals in the form of a two-phase course of post-transfusion immunological reactions. The first phase is characterized by inhibition of phagocytic activity of neutrophilic leukocytes and the HBT test index. The second one can be considered a stimulation phase. It is characterized by reverse immunological processes relative to the first phase. By stimulating the phagocytic and, specifically, oxygen-dependent bactericidal activity of neutrophil granulocytes in recipient rabbits.

Conclusions

It was found that allogeneic whole blood transfusion to recipient rabbits increases the phagocytic activity of blood neutrophils, with a simultaneous decrease in their absorption capacity.

On Days 3 and 7 after allogeneic whole blood transfusion to recipient rabbits, there is a significant decrease in the value of the spontaneous HBT test in the animal body, respectively, by 15% and 12.5%, which indicates inactivation of oxygen-dependent bactericidal activity of neutrophilic granulocytes during the first phase of post-transfusion immunological reactions.

On Day 23 after whole blood transfusion, a significant increase in the value of the spontaneous HBT test by 16.6% occurs in the body of rabbits, which indicates the activation of oxygen-dependent mechanisms of phagocyte killing and proves their bactericidal property.

It was found that on Day 3 after allogeneic whole blood transfusion, antibody-dependent cellular cytotoxicity decreases by 16% in the body of recipient rabbits. The authors of this study believe that the established pattern may be related to the synthesis of Class M immunoglobulins by plasma cells in the first days of the experiment, to which natural killers do not have specific Fc receptors.

On Day 7 of the experiment, 71.3% of antibody-dependent cellular cytotoxicity is significantly activated in recipient rabbits relative to the initial state. A probable increase in the value of this indicator indicates the activation of the synthesis of immunoglobulins of the secondary immune response (Ig G), to which natural killers (NK) have a complementary Fc receptor.

Antibody-dependent cytotoxic activity of lymphocytes on Day 23 after whole blood transfusion significantly increased by 61.5% in the body of recipient rabbits. The value of this indicator on Day 23 of the experiment in experimental animals was less by 5.6% relative to its Day 7, which occurs due to simultaneous natural apoptosis of allogeneic platelets and white blood cells within 7-16 days after whole blood transfusion.

Further studies will concern the investigation of the response of the cellular link of immunity (T, B, and 0 lymphocytes), in dynamics, in recipient animals by allogeneic transfusion.

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Вплив алогенної трансфузії крові на показники функціональної активності нейтрофілів і цитотоксичності лімфоцитів в організмі кролів-реципієнтів

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Анотація. Актуальність наукового дослідження зумовлена тим, що переливання алогенної крові тваринам-реципієнтам завжди пов'язане з імунологічними ризиками. У зв'язку з цим, метою роботи було оцінити стан фагоцитарної активності нейтрофілів крові за показниками фагоцитарного індексу, фагоцитарного числа та кисень-залежної бактерицидної активності, а також визначити зміни антитілозалежної цитотоксичної активності лімфоцитів у кролів-реципієнтів за алогенної трансфузії цільної крові. Моделювання переливання крові здійснювали на п'яти клінічно здорових кролях, шляхом внутрішньовенного введення їм цільної крові з розрахунку 5,5 мл/кг маси тіла. На 3, 7 та 23 добу після гемотрансфузії у тварин відбирали зразки крові. Зі зразків крові отримували популяцію нейтрофілів шляхом центрифугування на подвійному градієнті щільності 1,077 і 1,093 фіколу-верографіну. Поглинальну активність фагоцитів визначали у мікроскопічному тесті. Для вивчення кисень-залежної бактерицидної активності нейтрофілів проводили спонтанний тест з нітросинім тетразолієм. Колориметричним методом досліджували антитілозалежну цитотоксичну активність лімфоцитів. Встановлено, що після трансфузії цільної крові відбувається підвищення фагоцитарної активності нейтрофілів з одночасним зменшенням їх поглинаючої здатності. На 3 і 7 добу показники спонтанного тесту з нітросинім тетразолієм зазнавали зниження. Це свідчить про інактивацію кисень-залежної бактерицидної активності нейтрофільних гранулоцитів за першої фази посттрансфузійних імунологічних реакцій. На 23 добу відзначалося підвищення значень показників спонтанного тесту з нітросинім тетразолієм, що вказує на активацію бактерицидної властивості фагоцитів. З'ясовано, що на 3 добу антитілозалежна цитотоксична активність лімфоцитів вірогідно зменшувалася відносно вихідного стану, а на 7 і 23 добу – збільшувалася. Підвищення антитілозалежної цитотоксичної активності лімфоцитів слід пов'язувати з активним синтезом антитіл пізньої фази імунної відповіді. Отже, трансфузія алогенної крові спричиняє імунну відповідь у кролів-реципієнтів, не викликаючи при цьому негайних і віддалених трансфузійних реакцій (зміни частоти серцевих скорочень, частоти дихання, температури тіла). Отримані результати становлять практичну цінність як для науковців, так і для практикуючих лікарів, які застосовують переливання цільної крові та її компонентів тваринам за гострої анемії, порушенні функціональної активності факторів згортання крові, паразитарних і онкологічних захворюваннях

Ключові слова: лейкоцити, фагоцитарний індекс, НСТ-тест, антитіла, гранулоцити, імунітет

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Adenovirus infections in dogs: Diagnostic features

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Abstract. In the general pathology of dogs, viral diseases occupy a leading place, and infectious hepatitis, the causative agent of which is a virus of the *Adenoviridae* family, is of particular concern. Recently, the virus has spread to many countries around the world, which indicates changes in the properties of the pathogen. At the same time, the epizootic situation concerning infectious diseases, including adenoviruses, whose development is influenced by the mass keeping of dogs, has worsened in Ukraine. The purpose of this study is to supplement, clarify, and generalize data on epizootological features, morphological and biochemical parameters of blood and pathological and anatomical changes in type I canine adenovirus. The following research methods were used to conduct the research: epizootological analysis, clinical (determination of the general clinical condition of animals), pathological and anatomical (detection of macroscopic changes), haematological (morphological and biochemical parameters of blood) and statistical (processing of digital data to determine the probability of changes in indicators). According to the results of comprehensive studies, it was proved that dogs of different breeds, including mongrels, are susceptible to type I adenovirus, and the peak manifestation of the disease is the spring-summer period in animals aged one to two years. Infectious hepatitis is characterized by the development of erythrocytopenia, a decrease in haematocrit, leucocytosis, and lymphocytopenia. Changes in biochemical indicators are characterized by a decrease in haemoglobin, creatinine, urea, glucose in the blood, and a decrease in the activity of α-amylase and an increase in the content of total bilirubin, hyperfermentaemia of alkaline phosphatase and alanine aminotransferase. The most pronounced pathoanatomical changes are an increase in the size of the liver with the development of necrosis, and in the gastrointestinal tract-haemorrhages in the small intestine. The multisystem pathogenic effect of the virus is characterized by nephrosis, pinpoint haemorrhages in the pancreas, and inflammatory changes have been established in the lymph nodes. As a result of an experimental study of haematological parameters and pathoanatomical changes, a complex pathogenesis of the disease with multiple organ failure was established. In the study of infectious diseases of dogs, the most important aspect is clinical and diagnostic information content. Therefore, scientific research on a more profound understanding of the diagnosis of canine adenovirus will determine the algorithm for justifying the diagnosis

Keywords: infectious hepatitis, epizootological features, morphological and biochemical indicators of blood, erythrocytopoiesis, macroscopic changes

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Introduction

Since ancient times, man needed an assistant in the household and hunting, and the most suitable animal for this was a dog that was tamed over ten thousand years ago. Today, dogs are one of the most popular animal species and companions for humans, as evidenced by the increase in their number by 1.4% over the past decade (Ghasemzadeh & Namazi, 2015). It is established that more than 60% of European families have pets, most of which are dogs. Numerous studies have confirmed the significant and irreplaceable role of pets in human life and their positive impact on the psychosocial and mental health of their owners. Owning a pet can increase the activity of owners, reduce the risk of depression and mental stress, and increase self-esteem (Katagiri & Oliveira-Sequeira, 2008; Ghasemzadeh & Namazi, 2015). Therefore, an increase in the population of small animals, including dogs, has recently been recorded, which inevitably leads to an exacerbation of the epizootic situation (Netherton & Wileman, 2011; Radzikhovsky, 2016). Usually, diseases of infectious aetiology in dogs, in contrast to productive animals, do not lead to economic losses because hobby-class animals have a moral and psychological significance for their owners, and the death of a pet as a result of the disease can provoke a stressful state in them.

Many problems with the functioning of the gastrointestinal tract seem similar, but in reality, there are many aetiological factors that can lead to the death of an animal. Considering the data of professional literary sources, the authors of the present study state that in dogs, the main causes of the development of diarrhoea followed by symptoms of haemorrhagic enteritis are usually viruses, bacteria, and parasites (Rocha-Gizzi *et al*., 2014; Ortega *et al*., 2017; Lisova & Radsikhovskyi, 2018). On the territory of Ukraine, 60% of dogs are inpatient diagnosed with diseases with damage to the digestive system (Lisova & Radsikhovsky, 2018). The most common are viral enteritis, hepatitis, and gastroenteritis of unknown aetiology.

World literature sources indicate that the main causative agents of adenovirus infection in dogs are of two types: canine adenovirus type I (Rubart's disease, CAV-1), which causes canine infectious hepatitis, and canine adenovirus type 2 (CAV-2), which is associated with respiratory type canine adenovirosis. Types CAV-1 and CAV-2, which pertain to the *Adenoviridae* family, are closely related antigenically and genetically. Despite their antigenic and genetic affinity, they exhibit different patterns of cellular tropism. CAV-1 recognizes vascular endothelial cells and liver and kidney parenchymal cells as targets for viral replication, and CAV-2 efficiently replicates in the respiratory tract and, to a limited extent, in the intestinal epithelium (Decaro *et al*., 2008; Balboni *et al*., 2019; Zhu *et al*., 2022). One of the most frequent causes of gastrointestinal disorders in dogs is an infectious factor, which accounts for 63%, and aetiologically, these are viruses of the *Adenoviridae* and *Parvoviridae* families and bacteria of the *Salmonella* genus. It is worth separately noting the significant prevalence and, accordingly, the aetiological factor of the parasitic agent, namely *Giardia*, which was found in 37% of dogs with the corresponding symptoms. Of particular concern is the presence of mixed infection, i.e., the presence of two or more pathogens of different nosological groups in the body (Radzikhovskyi, 2016; Mitchell *et al*., 2017; Dowgier *et al*., 2018).

Adenoviruses affect the respiratory tract, gastrointestinal tract, and excretory system of animals. As a rule, they cause subclinical infection, but avian and canine adenoviruses cause a clinical manifestation of the disease. In animals with reduced immunity, the infection causes high morbidity and mortality. Adenovirus was first isolated in 1953 from culture explants of degenerated human adenoids by Wallace Rowe and his colleagues. Canine adenovirus belongs to the family *Adenoviridae* and the genus *Mastadenovirus* and is divided into canine adenovirus serotypes (Decaro *et al*., 2008 Timurkan *et al*., 2018; Musto *et al*., 2021).

Adenovirus infection of the first type (CAV-1), infectious hepatitis, Rubart's disease is an acute contagious disease characterized by fever, catarrhal inflammation of the mucous membrane of the respiratory tract and intestines, liver, and central nervous system damage. The disease is common in many countries of the world and occurs both among domestic animals and in the wild. Regular vaccination has reduced the number of clinical cases of the disease. The CAV-1 virus affects a considerable group of carnivores, including dogs of various breeds and ages, but most often puppies aged 2 to 6 months get sick. Therewith, the mortality rate is 30-40% and in the literature it is noted that female virus carriers can infect their puppies and males for many years (García-Marín *et al*., 2018; Hornsey *et al*., 2019; Yang *et al*., 2019).

Adenovirus type 2 is a highly contagious virus that damages the respiratory tract in dogs, especially puppies, when kept in conditions of considerable crowding. CAV-2 provokes disorders in the respiratory organs, including tonsillitis, pharyngitis, tracheitis, bronchitis, and having an affinity for the epithelium of the upper respiratory tract. This virus was first detected in Canadian shelter dogs in 1961 with symptoms of upper respiratory tract infection, and in the following years its presence was reported in many countries such as Italy, Brazil, and India (Hechinger *et al*., 2017; Ramidi *et al*., 2019; Bergmann *et al*., 2020).

Adenovirus infection in dogs is characterized by multifaceted clinical symptoms, but the main ones should be highlighted, namely lethargy, vomiting, dehydration, pallor, hypothermia, leukocytosis, neutrophilia, lymphopenia, and thrombocytopenia, increased activity of alkaline phosphatase, alanine aminotransferase, and creatine kinase. Over time, anaemia, hypoalbuminaemia, bilirubinaemia, bilirubinuria, haematuria, and proteinuria, splenomegaly, hepatomegaly, and pulmonary haemorrhage progress (Bulut *et al*., 2013; Pereira *et al*., 2021).

Based on the analysis of literature sources, the results of monitoring and own research, it was found out that viral diseases occupy a leading place in the infectious pathology of dogs and cause their mortality. And one of the most common is infectious hepatitis (Decaro *et al*., 2008; Radzikhovskyi, 2016; Mitchell *et al*., 2017). However, despite considerable progress in investigating the aetiopathogenesis of infectious diseases of animals and optimizing preventive measures, timely diagnosis of adenovirus infection in dogs stays an urgent issue on which the therapeutic effect depends.

The purpose of this study was to establish the nosological profile of viral diseases in dogs and to determine the share of adenovirus infection, as well as to conduct an analysis of morphological and biochemical indicators

of blood, the state of erythrocytopoiesis and pathomorphological changes to establish added diagnostic markers of this disease.

Materials and Methods

The study was conducted during 2021 at the Faculty of Veterinary Medicine at the National University of Life and Natural Sciences of Ukraine, as well as at the base of the veterinary clinics "Bahira" (Zhytomyr) and "Viktoria" (Kyiv). The study material was pedigreed and mongrel dogs with adenovirus infection.

During the study, the main rules of good laboratory practice (GLP) (On approval of the Procedure..., 2009), the provisions of the "General ethical principles of animal experiments" adopted by the First National Congress of Bioethics (Kyiv, 2001) were followed (On the protection..., 2021). The entire experimental part of the study was conducted per the requirements of the international principles of the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Purposes" (Strasbourg, 1986) (European Convention..., 1986), "Rules for Conducting Work Using Experimental Animals", approved by the Order of the Ministry of Healthcare No. 281 dated November 1, 2000 "On Measures to Further Improve Organizational Forms of Work with the Use of Experimental Animals" and the corresponding Law of Ukraine "On the Protection of Animals from Cruelty" (No. 3447-IV dated 02/21/2006, Kyiv) (Law of Ukraine..., 2006). Euthanasia was performed using drugs that ensure rapid, painless death, according to the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Purposes" (European Convention..., 1986).

At the first stage of research, the epizootic situation regarding infectious diseases of dogs in the service area of veterinary medicine clinics was analysed. For this, the authors of this study used the primary documentation of outpatient logs of animal admissions. At the same time, the anamnestic data presented by the owners of dogs with a confirmed diagnosis or suspicion of adenovirus infection were additionally considered.

During the analysis of the documentation regarding the epizootological features of the course of the disease for the year 2021, the presence of viral pathogens was found and confirmed in 105 dogs, and adenovirus infection in 60 animals.

Diagnostic studies to confirm adenovirus were conducted using VetExpert express test systems (CAV Ag, solid phase immunochromatographic analysis for qualitative detection of *Canine Adenovirus* antigen) manufactured in Poland. Diagnostic studies on the presence of adenovirus antigen in dogs were also conducted at the private veterinary laboratory Bald LLC (Kyiv) using enzyme-linked immunosorbent assay and polymerase chain reaction. Morphological and biochemical indicators of blood were studied using a BioChem SA (USA) biochemical analyser using High Technology, Inc. reagents (USA).

At the second stage of research, haematological studies were conducted and indicators of erythrocytopoiesis in the blood of dogs (n = 21) with adenovirus infection were determined. The control group included clinically healthy dogs (n = 10).

General clinical blood analysis included the determination of the number of erythrocytes and leukocytes by the melanger method in a chamber with a Goryaev grid; haemoglobin content in blood – haemoglobin cyanide method; haematocrit value – by Shklyar microcentrifugation. Based on the obtained results, red blood indices were calculated – the haemoglobin content in one erythrocyte (MCH), the average concentration of haemoglobin in an erythrocyte (MCHC), and the average volume of erythrocytes (MCV).

During the biochemical examination of blood serum, the following were determined: the content of total protein – refractometrically, the concentration of urea – by colour reaction with diacetyl monooxime, and creatinine – by the Jaffe method, the activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) by the Reitman-Frankel method. Blood for research was collected from dogs from the superficial vein of the forearm *v. Anterbrachium* and medial or subcutaneous vein of the lower leg *v. Saphena*.

To determine the replication and persistence of adenovirus, a pathoanatomical study of dogs of different ages who died from infectious hepatitis was conducted. Pathoanatomical autopsy of dog corpses was performed in the dorsal position, by partial evisceration in the generally accepted sequence. Each time, the autopsy was recorded, and the detected changes were photographed with a Nikon camera (Indonesia).

Digital data were biometrically processed according to generally accepted methods of variational statistics using the computer software Statistica 6.0 and Microsoft Excel 2007. The probability of differences between the values of indicators was evaluated according to the Student's t-test. Three degrees of probability were calculated: *P < 0.05; **P < 0.01; ***P < 0.001.

Results and Discussion

To find the epizootic situation regarding adenovirus infection and its prevalence, epizootic monitoring was conducted, and the level of morbidity of dogs with various viral diseases was analysed. Based on the results obtained, a nosological profile of viral diseases of dogs was formed (Fig. 1).

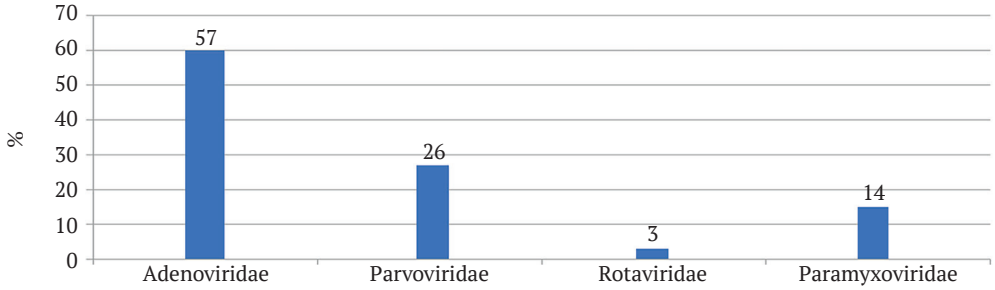


Figure 1. Nosological profile of viral diseases of dogs in 2021, %

In 2021, pathogens of viral infectious diseases were identified and confirmed in 105 dogs as follows: adenovirus – 57%, parvovirus – 26%, rotavirus – 3%, and paromyxovirus – 14% (Fig. 1). The obtained results coincide with the data of S. A Headley *et al.*, (2018), which note the tension of the epizootological situation regarding viral diseases of dogs and the rapid spread of infectious ones, with a growing trend not only in Ukraine, but also throughout the world. Therewith, a special place is occupied by adenovirus infection, which is described in Ukrainian and foreign scientific publications (Ortega *et al.*, 2017; Lisova & Radsikhovskyi, 2018; Ji *et al.*, 2020).

The initial diagnosis of infectious diseases begins with an epizootological analysis, which highlights the patterns of infectious disease development. During the study of epizootological features, certain factors were considered – the migration of animal owners with their animals to other clinics of veterinary medicine, as well as the pedigree, seasonality, and age-related susceptibility to viral diseases were determined by analysing the data of 44 dogs infected with adenovirus. Thus, the most susceptible to adenovirus infection of the first type are mongrel dogs – 12 (27%), and among pure-bred dogs: Dobermanns – 9 (20%), Boxers – 8 (18%), and Rottweilers – 5 (11%). Adenovirus was considerably less frequently diagnosed in German shepherds, Kurtzhaars, and Great Danes (5%) and the lowest number of cases (2%) was found in Shepherds (Asian and European), Spaniels and Dalmatians. Adenovirus infection manifests itself seasonally, specifically in the spring-summer period; it accounts for 27 to 40% of cases, which corresponds to the pattern of the peak manifestation of viral diseases. During this period, there is close contact between animals during longer walks and exhibitions, which is facilitated by the climatic conditions of Ukraine.

One of the important criteria for assessing epizootological features is the determination of age-related predisposition to adenovirus infection. Therewith, the highest rate was diagnosed in dogs aged one to two years – 26%, in puppies and dogs aged up to one year, and from two to five – 21%, and in animals aged two to six months – 18%. Adult animals over the age of five are susceptible to this disease in 18%. In most infectious diseases, maternal (colostral) antibodies play a significant role, and adenovirus infection is no exception, which was not diagnosed in puppies under two months of age according to this study.

Two forms of manifestation of adenovirus infection were established – intestinal, which is characterized by damage to the gastrointestinal tract, and adenovirus of the first type acts as an antigen (Fig. 2 A, B), and specific – haemorrhagic diarrhoea, vomiting, jaundice and dehydration, sometimes ascites developed. In case of adenovirus of the second type, damage to the respiratory organs was established with the development of a corresponding symptom complex, the specific manifestation of which is the occurrence of keratitis, called "blue eye" (Fig. 2 C). Notably, eye damage and the manifestation of uveitis is observed in adenovirus type I. At the same time, Zhu *et al.*, 2022 indicate that CAV-1 and CAV-2 are closely related antigenically and genetically.

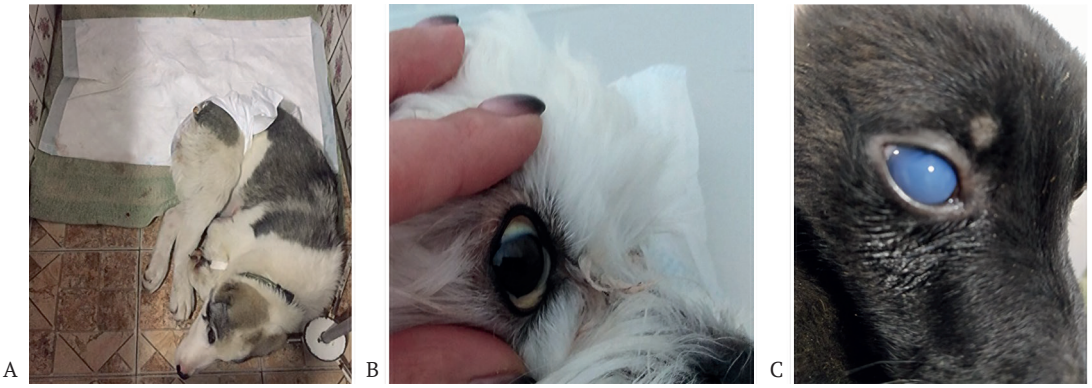


Figure 2. Main clinical symptoms in dogs with adenovirus

Note: *A – haemorrhagic enteritis and lethargy due to CAV-1; B – jaundice of the visible mucous membrane of the eyes due to adenovirus CAV-1; C – uveitis due to CAV-1 and CAV-2*

Analysing the results of the authors' earlier research and studies presented in specialized literature sources (Hornsey *et al.*, 2019; Thompson *et al.*, 2020; Musto *et al.*, 2021), it was established that the respiratory form of adenovirus infection has a benign course, the mortality rate does not exceed 10%, and it is effectively treated according to generally established protocols. But the intestinal form often leads to generalization of the pathological process and is characterized by a considerable mortality rate of 40%. Therefore, for a more detailed definition of the specific features of the physiological state of animals under adenovirus type I, changes in the morphological composition of the blood were subject to investigation (Table 1). Blood parameters are sensitive and informative indicators of the state of the body, undergo changes due to the action of both exogenous and endogenous factors. During the experimental study, the manifestation of an intestinal form of adenovirus with characteristic symptoms of gastrointestinal tract damage was established in 21 out of 39 animals.

Table 1. Morphological parameters of the blood of dogs with adenovirus, M ± m

Indicator		Clinically healthy animals (control group), n = 10	Animals infected with adenovirus (experimental group), n = 21
Red blood cells (RBC), 10^{12}/l		6.1 ± 0.4	4.3 ± 0.6*
Haematocrit value (HCT), %		45.4 ± 2.4	27.9 ± 1.7***
Platelets (PLT), 10^9/l		428.1 ± 11.2	343.6 ± 34.1*
White blood cells (WBC), 10^9/l		9.4 ± 0.2	19.2 ± 1.3***
Neutrophils (NEU), %	J	0	–
	S	58.5 ± 1.4	78.6 ± 1.8***
	B	1.7 ± 0.2	1.6 ± 1.2
Eosinophils (EO), %		1.8 ± 0.3	1.2 ± 0.1
Monocytes (MON), %		3.0 ± 0.2	1.2 ± 0.3***
Lymphocytes (LYM), %		35.0 ± 1.3	18.6 ± 3.2***
ESR, mm/h		4.4 ± 0.5	17.0 ± 2.2***

Note: **P < 0.05; **P < 0.01; ***P < 0.001, significant compared to the control group*

According to the results of the haematological studies (see Table 1), it was found that with adenovirus infection of the first type in the blood of dogs, the number of erythrocytes significantly decreases by 29% (P<0.05) compared to animals of the control group. It is assumed that a decrease in the number of red blood cells in the blood of dogs infected with adenovirus stimulates the development of an infectious agent and causes their haemolysis. It was established that the haematocrit value is probably lower by 17.5% (P < 0.001) compared to that of healthy animals and, considering erythropenia, may indicate the development of anaemia. A probable decrease in the number of platelets in the blood by 20% (P < 0.05) is associated with their destruction in the liver and spleen due to the features of the pathogenic influence of adenovirus. A pathological increase in the number of leukocytes in the blood by 2 times (P < 0.001) occurs under an infectious agent, which stimulates the formation of leukocytes and is a protective reaction of the body. In adenovirus infection, neutrophilic leucocytosis was established as a result of a probable increase of 20% (P < 0.001) in the number of segmented neutrophils, which indicates a shift of the leukocyte formula to the right and characterizes anaemia and the development of hepatorenal syndrome. In relation to the significant decrease in the number of monocytes by 2.5 times (P < 0.001), this fact may indicate not only the onset of anaemia, but also bone marrow damage. A probable decrease of 1.9 times (P < 0.001) in the number of lymphocytes was also established, which distinguishes infectious processes with an acute course. Lymphocytopenia was probably caused by low molecular weight proteins – interferons, which are synthesized in response to a viral infection. The rate of sedimentation of erythrocytes in the blood of infected animals probably increased by 4 times (P < 0.001) compared to a similar indicator in healthy animals. This fact is possibly related to an increase in plasma concentration of acute phase proteins, namely fibrinogen, C-reactive protein, and immunoglobulins (Table 1).

The results of biochemical studies of the blood serum of dogs with adenovirus infection type I did not differ significantly from similar indicators in clinically healthy animals (Table 2). Therewith, considering the "red blood" indices, they characterize the intensity of maturation of red blood cells and their saturation with haemoglobin in the bone marrow due to adenovirus hepatitis.

Table 2. Biochemical indicators of dog blood serum with adenovirus, M ± m

Indicator	Clinically healthy animals (control group) n = 10	Animals infected with adenovirus (experimental group), n = 21
Haemoglobin content in one red blood cell (MCH), pg	23.6 ± 1.6	22.3 ± 1.8
Average concentration of haemoglobin in erythrocytes (MCHC), g/dl	33.9 ± 1.4	33.5 ± 1.7
Average red blood cell volume (MCV), fl	67.1 ± 3.7	66.1 ± 2.8
Haemoglobin (HGB), h/l	145.0 ± 12.5	93.1 ± 7.2**
Glucose, mmol/l	4.2 ± 0.2	1.7 ± 0.4***
Total bilirubin, μmol/l	0.22 ± 0.2	2.8 ± 0.3***
Alkaline phosphatase (ALP), IU/l	34.6 ± 2.7	74.8 ± 3.4***
Total protein, g/l	71.0 ± 1.8	69.6 ± 8.4
Aspartate aminotransferase (AST), IU/l	50.1 ± 3.8	46.7 ± 4.1
Alanine aminotransferase (ALT), IU/l	46.6 ± 3.3	113.8 ± 6.4***
De Ritis coefficient, IU/l	1.24 ± 0.2	0.42 ± 0.3**
Urea, mmol/l	5.3 ± 0.2	2.2 ± 0.4***
Creatinine, μmol/l	97.0 ± 3.9	48.8 ± 7.2***
(α-Amylase), IU/L	1120.0 ± 65.4	205.0 ± 15.2***

Note: **P < 0.05; **P < 0.01; ***P < 0.001, significant compared to the control group*

Notably, according to the indicators presented in the Table 2, it is possible to state the development of microcytic hypochromic iron deficiency anaemia. Furthermore, this is confirmed by a significant decrease in haemoglobin content by 36% (P<0.01) compared to the control. Adenovirus infection was diagnosed as a violation of the functional state of the liver, as evidenced by changes in such biochemical indicators as a probable decrease in urea concentration by 2.4 times (P < 0.01), the De Ritis coefficient by 2.95 times, and an increase in the level of total bilirubin by 12.7 times (P < 0.001). At the same time, there is a 2.4-fold increase in ALT activity in the blood serum (P < 0.001), which characterizes the development of cytolysis of liver cells. A probable increase in the activity of alkaline phosphatase by 2.2 times (P < 0.001) with a simultaneous decrease in the activity of α-amylase by 5.5 times (P < 0.001) was also established, which characterizes the development of hepatopathology in infected dogs.

Creatinine concentration is an important indicator of kidney activity, especially their filtration capacity. During adenovirus infection, this indicator was characterized by probably 2 times lower values (P < 0.001) than in healthy animals, which also indicates changes in muscle mass due to exhaustion of the body.

When diagnosing adenovirus in dogs, the concentration of glucose in the blood serum should be analysed because this carbohydrate is a vital energy substrate, which in infectious hepatitis acquires values significantly lower by 2.5 times (P < 0.001) than in healthy animals. The authors of the present study believe that this fact characterizes hypoglycaemia due to the occurrence of hepatorenal syndrome and damage to the gastrointestinal tract. In animals with low blood glucose levels, there is a compensatory increase in gluconeogenesis, which is primarily inherent in the liver, as well as to some extent the cortical layer of the kidneys and the epithelium of the small intestine (Table 2).

Thus, the results of the analysis of morphological and biochemical parameters of dog blood indicate a multisystem effect of adenovirus infection of the first type on their body. The obtained results, namely anaemia and an increase in the activity of liver enzymes in the blood serum and changes in indicators characterizing the damage to the renal system, coincide with the data of other scientists (Verin *et al*., 2019; Polovitzer *et al*., 2022).

For a comprehensive study of the deceased animal, it is necessary to conduct an autopsy with clarification of the correctness of the lifetime diagnosis. Therewith, the cause of death of animals can be determined by pathological and morphological changes in the organs. Organ damage caused by adenovirus infection of the first type depends on the duration and severity of the disease. Usually, the corpses of puppies that have died from an adenovirus infection are depleted and anaemic. Their serous integuments are dry. During an autopsy, a considerable amount of clear or serous-haemorrhagic fluid was found in the abdominal cavity of dog corpses. Haemorrhages and catarrhal inflammation of the duodenum were observed on the mucous membrane of the small intestine, and haemorrhagic foci were observed in the thymus and pancreas. The lymph nodes were enlarged, hyperaemic, and swollen. The spleen was enlarged, dense, and fibrinous perisplenitis (inflammation of the connective tissue capsule covering the spleen) with central pyogranulomatous necrotic inflammation (splenitis) was observed, sometimes haemorrhages and infarctions were found on the surface (Fig. 3).

In isolated cases, the mucosa of the small intestine was light pink, without damage or necrosis. The surface of the villi epithelium is destroyed. The contents of the intestines are liquid, the faecal masses had an unpleasant smell, their colour ranges from dirty yellow to dark red (Fig. 4).

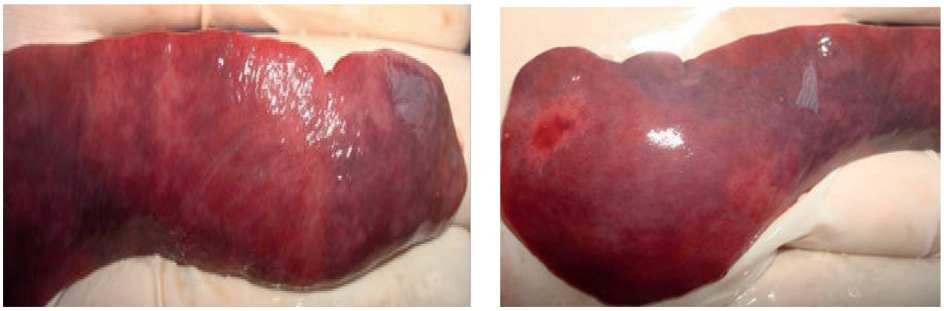


Figure 3. Macroscopic changes in the spleen of a dog with adenovirus type I

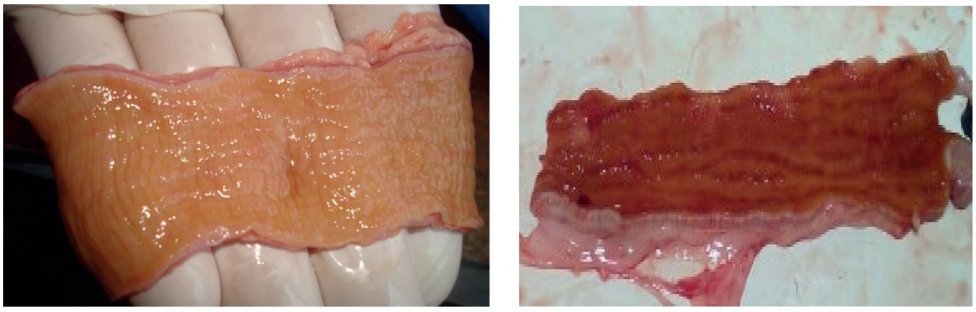


Figure 4. External view of a section of a fragment of the small intestine of a dog with adenovirus type I

The faeces contained mucus. Haemorrhagic enteritis has been established in most animals due to adenovirus infection. Macroscopic changes of the liver in adenovirus infection type I are considered pathognomonic, namely an increase in size (hepatomegaly), a yellowish-brown colour, full blood with isolated haemorrhages and rounded areas of necrosis, a loose consistency, which collapses under slight pressure. The gallbladder is grey green, swollen, and thickened, pear-shaped, without damage, filled with bile inside, which to some extent coincides with the results of research by M. Wong *et al*. (2017) (Fig. 5).

During the development of light pink lung disease, heterogeneous areas of consolidation due to bronchopneumonia are noticeable. The consistency of the organ is doughy, loose to the touch and with the phenomena of pneumonia, atelectasis of the lungs is noted (Fig. 6).

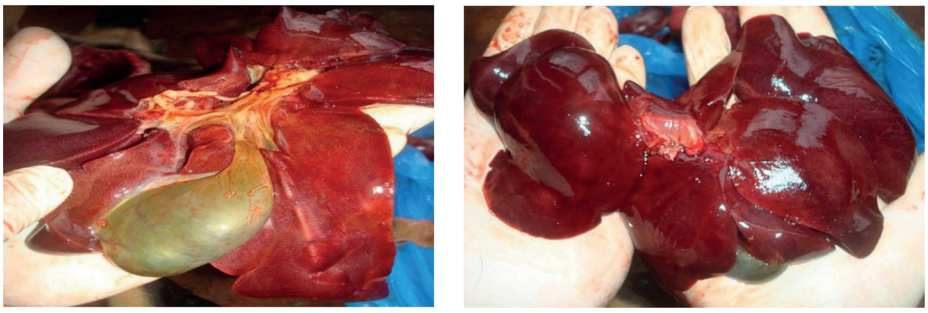


Figure 5. Macroscopic changes in the liver and gallbladder of a dog with adenovirus type I

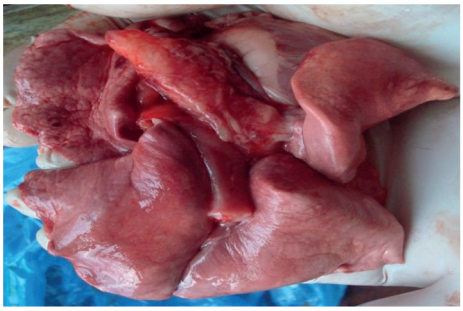
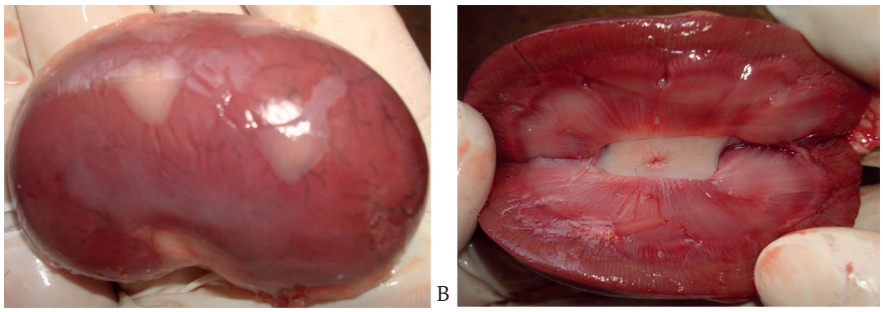


Figure 6. Macroscopic view of the dog's lungs with adenovirus type I

Kidneys are smooth, single papilla, have a bean-like shape, grey-red colour, flabby consistency (Fig. 7 A). The capsule is easily removed. On the section (Fig. 7 B), depending on the duration of the disease, the pattern is from weakly expressed to indistinct, the edges of the section do not converge. Some animals were diagnosed with capsule haemorrhages and nephrotic changes.

Preyß-Jägeler *et al*. (2022) note that infectious agents can cause glomerulopathy and acute kidney injury and are a common complication and a common cause of death in dogs.



A B

Figure 7. Macroscopic view of the dog's lungs with adenovirus type I

In case of adenovirus infection type I in dogs, the pancreas is slightly enlarged, hyperaemic and blood-filled with pinpoint haemorrhages.

Pathological and anatomical changes were also found in the lymph nodes, namely serous lymphadenitis with individual or multiple point haemorrhages both from the surface and on the incision. Therewith, the colour of the lymph nodes ranges from greyish red to dark cherry.

Conclusions

This paper summarizes the authors' own and international experience concerning the main aspects of epizootological features, clinical manifestation and pathological and morphological changes upon canine adenovirus infection type I. It was established that among viral diseases, adenovirus antigen was diagnosed in 57% of dogs under study, with specific clinical symptoms of damage to respiratory organs

and disorders of the gastrointestinal tract. Epizootologically, adenovirus type I is characterized by spring-summer seasonality with a frequency of occurrence in dogs from 27% to 40%, and the most pronounced breed predisposition in Dobermanns, Boxers, and Rottweilers aged from one to two years (21%). One of the specific clinical symptoms of infectious hepatitis B in dogs is disorders in the gastrointestinal tract and liver, which are manifested by haemorrhagic diarrhoea and jaundice. A supplementary and specific marker of the clinical manifestation of adenovirus is eye pigmentation, the so-called "blue eye".

The development of microcytic hypochromic iron deficiency anaemia was established based on the results of a decrease in the number of red blood cells in the blood by 29%, haematocrit value – by 17.5% and haemoglobin content – by 35-40%. Therewith, a significant increase in the total bilirubin content in blood serum should be noted – by 12.7 times, alkaline phosphatase activity – by 2.2 times, and ALT – by 2.4 times, and a decrease in urea concentration – by 2.4 times, which indicates the development of hepatitis. This is confirmed during an autopsy and is a pathognomonic symptom. A 2-fold decrease in creatinine concentration indicates a reaction of the renal system and considering neutrophilic leucocytosis with a shift in the leukocyte formula to the right, the development of hepatorenal syndrome. A 2.5-fold decrease in the concentration of glucose in the blood serum was also found, which indicates the development of hypoglycaemia, inherent in diseases of the gastrointestinal tract with the manifestation of haemorrhagic enteritis.

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Аденовірусні інфекції у собак: особливості діагностики

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Анотація. У загальній патології собак вірусні хвороби посідають провідне місце, а особливе занепокоєння викликає інфекційний гепатит, збудником якого є вірус родини *Adenoviridae*. За останні роки вірус поширився в багатьох країнах світу, що свідчить про зміни властивостей збудника. Водночас і в Україні загострилася епізоотична ситуація щодо інфекційних хвороб, включаючи аденовірусні, на розвиток яких впливає масове утримання собак. Метою роботи є доповнення, уточнення та узагальнення даних щодо епізоотологічних особливостей, морфологічних і біохімічних показників крові та патологоанатомічних змін за аденовірозу собак першого типу. Для проведення досліджень були використані такі методи досліджень як: епізоотологічні (епізоотологічний аналіз), клінічні (визначення загального клінічного стану тварин), патологоанатомічні (виявлення макроскопічних змін), гематологічні (морфологічні та біохімічні показники крові) та статистичні (обробка цифрових даних з метою визначення вірогідності змін показників). За результатами проведених комплексних досліджень доведено, що до аденовірозу першого типу сприйнятливі собаки різних порід, включаючи безпородних, а пік маніфестації хвороби – це весняно-літній період у тварин від одного до двох років. Для інфекційного гепатиту характерним є розвиток еритроцитопенії, зниження гематокритної величини, лейкоцитоз та лімфоцитопенія. Зміни біохімічних показників характеризуються зменшенням у крові вмісту гемоглобіну, креатиніну, сечовини, глюкози і зниженням активності α-амілази та збільшенням умісту загального білірубіну, гіперферментемією лужної фосфатази та аланінамінотрансферази. Найвираженішими патологоанатомічними змінами є збільшення розмірів печінки з розвитком некрозу, а у шлунково-кишковому тракті – крововиливи в тонкому відділі кишечнику. Мультисистемний патогенний вплив вірусу характеризується нефрозом, точковими крововиливами у підшлунковій залозі, а у лімфатичних вузлах встановлено зміни запального характеру. В результаті експериментального дослідження гематологічних показників та патологоанатомічних змін встановлено складний патогенез хвороби з поліорганною недостатністю. У вивченні інфекційних хвороб собак найважливішою є клініко-діагностична інформативність. Тому, наукові пошуки щодо глибшого розуміння діагностики аденовірозу собак визначать алгоритм обґрунтування постановки діагнозу

Ключові слова: інфекційний гепатит, епізоотологічні особливості, морфологічні та біохімічні показники крові, еритроцитопоез, макроскопічні зміни

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Cytomorphological characteristics of necroptates of internal organs of dogs in the early post-mortem period in the aspect of forensic veterinary examination

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Abstract. The relevance of the study lies in the need for forensic veterinary examination of animal corpses for scientific justification of informative diagnostic criteria for assessing the prescription of death, especially in the early post-mortem period. However, information about early post-mortem changes in dog cadavers at the microstructural level in the Ukrainian and foreign scientific literature is quite fragmentary. In this regard, the purpose of this paper is to establish the informative dynamics of the processes of cell destruction and bacterial contamination of internal organs of dog corpses during the first post-mortem day to establish probable expert criteria for the prescription of death of sub-expert animals during the forensic veterinary examination. A leading approach to the investigation of this problem is the method of obtaining a series of necroptates from lungs, heart, liver, spleen, kidney, pancreas, and brain from canine cadavers, over the same time interval during the first day after death. In cytological preparations obtained from necroptates, the number of destroyed cells and bacterial units was counted using optical microscopy. Based on the results of the dynamics of bacterial contamination and the intensity of morphological changes in spleen and pancreatic cells, their expert information content was established to solve the question of the prescription of death of dogs, regardless of weight and fatness indicators. It was found that the dynamics of bacterial contamination and cellular destruction of the brain, kidneys, and lungs of dog corpses have average expert information content, while the liver and heart are not informative. It was proved that the dynamics of destructive post-mortem processes in the cells of the compact organs of the corpses of dogs of different weight and fatness at the appropriate times probably do not differ and develop with the same intensity. The obtained results of the study will have significance both in the theory of forensic veterinary examination and directly applied, specifically when the forensic expert solves the question regarding the time limit for the death of the animal

Keywords: subexpert animals, cadaveric biotransformation, cell destruction, bacterial contamination, post-mortem organ changes

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Introduction

Forensic veterinary examination is a new type of forensic examination in Ukraine, which was established in 2019 at the initiative of the National Research Centre "Ex. Prof. M.S. Bokarius Institute of Forensic Examinations" in the system of specialized state institutions of the Ministry of Justice of Ukraine (Klyuyev, 2019).

During a forensic veterinary examination, only a forensic veterinary expert is the subject of expert activity, who is authorized by law to resolve issues related to the identification and assessment of bodily injuries caused to an animal as a result of cruel treatment (Yatsenko & Parilovskyi, 2020; Randour *et al*., 2021), during a traffic accident (Conroy *et al*., 2019), poaching (Franckenberg *et al*., 2015), in case of violation of their exploitation regime (Hall *et al*., 2020), use in various spectacular events (Doelling *et al*., 2021) etc. One of the objects of forensic veterinary examination is the cadaver of an animal (Ang *et al*., 2019; Yatsenko & Kazantsev, 2021), examining which the forensic expert has to solve various questions, including regarding the time of its death (Yatsenko *et al*., 2019).

Modern practice and tactics of forensic veterinary diagnostics in Ukraine require scientific substantiation of informative diagnostic criteria at the level of European forensic veterinary science (Parry & Stoll, 2020), namely regarding the assessment of the age of death of animals, primarily in the early post-mortem (antemortem) period. This is especially important for the most correct and precise establishment of the post-mortem interval and can substantially affect the further course of the investigation of crimes against animal life and health (Listos *et al*., 2018).

The problem of determining the statute of limitations for the occurrence of animal deaths stays relevant for forensic veterinary experts, law enforcement agencies, and the court, which has been repeatedly discussed at scientific forums of various levels both in Ukraine and abroad (Munro *et al*., 2020).

Currently, the most accessible method of determining the statute of limitations for the occurrence of animal death in forensic veterinary examination is a visual assessment of post-mortem changes, which are a macroscopic manifestation of biochemical and biophysical processes occurring in the animal's body after death. These include studies of cadaver spots, cooling, desiccation, post-mortem rigidity, supravital reactions, etc. They are quite informative, have important diagnostic forensic value, but do not always allow reliably and reasonably accurately establishing the duration of the post-mortem period of an animal corpse. This is explained by the fact that the natural changes that occur in the animal's body after death are complex and mostly unpredictable, since the emergence and development of post-mortem phenomena is influenced by a considerable range of factors (Touroo & Fitch, 2016).

The accuracy of visual methods for investigating an animal's corpse requires improvement. Therefore, the focus of forensic veterinary experts is shifted towards cytological and biochemical methods, which are based on systematic pathophysiological changes and are more precise and objective compared to such external factors.

At the current stage of the development of forensic veterinary expertise, scientists have developed and implemented into expert practice differential diagnostic criteria for determining the age of death of animals based on a limited range of signs (Listos *et al*., 2018; Cao *et al*., 2021). However, in forensic humane medicine, there are already many proven methods that give positive results and help in obtaining answers to numerous questions that have not yet been resolved. However, even the proposed latest research methods cause obstacles in their adaptation to forensic practice due to problems of material and technological support.

One of the options for solving this problem is to increase the accuracy and objectification of the structural and functional changes in the internal organs of the animal body of different morphological types in the post-mortem period (Ceciliason *et al*., 2021).

A prominent place in forensic and veterinary thanatology is occupied by cytomorphological studies, the results of which can be used to establish the time of death of animals (Wunsche *et al*., 2016). These issues are periodically discussed on the pages of scientific periodicals of Ukraine (Kazantsev & Yatsenko, 2021). However, many issues are still unresolved, including the cytomorphological characteristics of necroptates of internal organs of dogs in the early post-mortem period in the aspect of forensic veterinary examination. Thus, the issue under study is relevant from the standpoint of the practice of forensic veterinary expertise.

Therefore, the purpose of this study was to provide cytomorphological characteristics of necroptates of internal organs of dog cadavers in the early post-mortem period and to substantiate its informativeness for establishing the time of death during a forensic veterinary examination. Such studies were conducted in Ukraine for the first time.

Literature Review

The possibilities of the classic method of examining animal cadavers – forensic autopsy, due to the frequent absence of macroscopic changes in the antemortem and early post-mortem period, are limited. This predetermines the need for forensic experts to use the latest research methods. Thus, Heng *et al*. (2009) described serial post-mortem radiographic data of the abdominal cavity in cadavers of dogs; Listos *et al*. (2016) provided a post-mortem estimate of time of death based on renal versus rectal temperature measurement; Panasiuk-Flak *et al*. (2021) proved the informativeness of the method of determining the time of death of dogs using atropine and pilocarpine in the early post-mortem period; Listos *et al*. (2017) demonstrated the possibility of estimating the time of death of animals by the development of microflora in the calf muscle of a dog; Li *et al*. (2020) provided molecular characterization of intestinal microbial shift in rats after death for 30 days; Paltian *et al*. (2019) described a procedure for estimating the post-mortem interval by determining the activities of catalase and delta-aminolevulinate dehydratase in liver, kidney, skeletal muscle, and brain tissues of mice; Swiss *et al*. (2022) investigated post-mortem changes in the electrical conductivity

of *Dicentrarchus labrax* skeletal muscles to estimate the post-mortem period; Dell'Annunziata *et al*. (2022) characterized the post-mortem interval using MALDI-TOF mass spectrometry analysis in mouse cadavers; El-Din *et al*. (2021) assessed post-mortem skin changes on animal and human cadavers to estimate time since death; Welson *et al*. (2021) determined the duration of death in male albino rats by investigating markers of oxidative stress, histopathological and molecular changes in internal organs; Zhang *et al*. (2022) established the statute of limitations for the occurrence of death of rats in the case of drowning by the composition of vitreous metabolites; Grela *et al*. (2018) substantiated the possibility of using imaging methods of sectional research in forensic veterinary medicine.

However, these and other proposed methods for determining the post-mortem interval require the use of appropriate equipment, special skills of forensic experts who use them, which is not always possible in everyday forensic veterinary practice.

A separate place in the diagnostic process is occupied by the analysis of microstructural (histological and cytological) changes in the internal organs of the animal corpse, which are a reflection of the biochemical and biophysical processes occurring in it after death, especially in the early post-mortem period, when its morphological changes are still invisible to the naked eye (Brooks Brownlie & Munro, 2016).

However, fragmentary studies of foreign scientists on histological changes in the internal organs and skeletal muscles of animal corpses of various species in the early post-mortem period do not cover the issue sufficiently.

Thus, the clarification of cytomorphological changes in the internal organs of dog corpses in the early post-mortem period in the practice of forensic veterinary expertise will be important both in the theory of forensic veterinary expertise, and directly applied, specifically when a forensic expert decides on the statute of limitations for the occurrence of death of a sub-expert animal.

Materials and Methods

The study was conducted during 2021-2022 in the conditions of the Department of Sanitation, Hygiene, and Forensic Veterinary Medicine of the State Biotechnological University (Kharkiv).

For a randomized study, cadavers of domestic dogs (*Canis familiaris*, n = 14) of the same age (3-4 years) were selected and divided into two experimental groups according to the principle of analogues in terms of their weight, degree of fattening, and in the absence of *anamnesis morbi*. All cadavers, according to the Law of Ukraine "About by-products of animal origin, not intended for human consumption" (Law of Ukraine No. 1089-IX..., 2020), are classified as category III by-products, safe for humans and other animals.

Group 1 included cadavers of dogs (n = 7) with a weight of 8.0 ± 1.0 kg, fatness above average. Other cadavers of dogs (n=7) with below-average fatness and body weight of 16.0 ± 1.0 kg were selected for Groups 2. After the same time interval (3 hours) during the first day after death, a series of necroptates from the lungs, heart, liver, spleen, kidneys, pancreas, and brain in the size of 1 × 1 × 1 cm^3 were taken from the corpses of dogs of both groups. The study was conducted on the cadavers of dogs during the first day after the animal death before the emergence of late cadaveric phenomena, i.e., in the early post-mortem period. Native smears-imprints were made from each necroptate. The slides without prior fixation were immediately dried in air at 20°C. The obtained cytological preparations were stained according to the Pappenheim method in the A.I. Kryukov's modification, which provides the possibility of accelerated differentiation of structural elements of internal organs and their changes in smears-imprints at the cellular level (Zaporozhan *et al*., 2002).

Microscopic examination of the obtained cytological preparations was performed using an optical binocular microscope Granum R50 (China) in the fields of view of 10 × 10 and 10×100. Cytograms were obtained using a ToupCam UCMOS03100KPA digital camera (China), which were analysed at the final stage of the experiment, considering the recommendations of Ressel (2017).

In cytological preparations, the number of destructively changed cells and bacteria was counted separately in ten fields of view of the microscope, and then their average was determined.

The obtained digital data was statistically processed using a personal computer, using the software package "MS Office Excel 2016 (Statistica)", by calculating the arithmetic mean (M ± m) for each of the two indicators. The results of the study were evaluated by the level of significance (P). The dynamics and intensity of cell destruction and bacterial contamination of the internal organs of dog cadavers in each group were compared for the previous and subsequent time intervals, as well as between each other in the intervals when they were first detected and at 24 hours of the experiment.

Results and Discussion

The cytoarchitectonics of smears-imprints obtained from the tissues of the internal organs of dog cadavers, immediately after the onset of their death, preserves the usual lifetime condition. Thus, pneumocytes, cells of the ciliated epithelium, and neutrophils are well visualized on the cytograms of the lungs of the cadavers of dogs of both experimental groups. Nuclear cells present in large numbers are mainly well-differentiated respiratory epithelial cells. Pneumocytes (Fig. 1) and neutrophils (Fig. 1) are located discretely or in small clusters. They are spherical or oval. Their cytoplasm is grey, with a few optically transparent vacuoles. The cell nucleus contains compact chromatin, and the nucleolus is visualized in individual cells. The background of the cytogram is represented by a population of unchanged neutrophils. Ciliated epithelium of the lungs (Fig. 2) the cadavers of dogs of the EG 2 are located in small clusters. The cytoarchitecture of its cells is bipolar: at the basal pole there is a single nucleus with fine-grained chromatin and a nucleolus without signs of morphological changes. Pink cilia are visible at the apical pole.

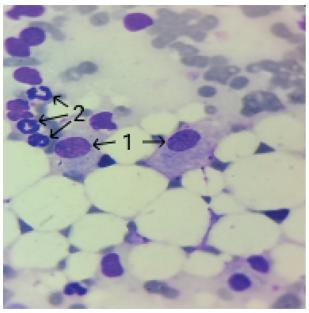

Figure 1. Cytogram of the lungs of the corpse of a dog of the EG 1 immediately after death. Eyepiece 10×lens 100, stained according to Pappenheim-Kryukov
Note: *1 – pneumocytes; 2 – neutrophils*

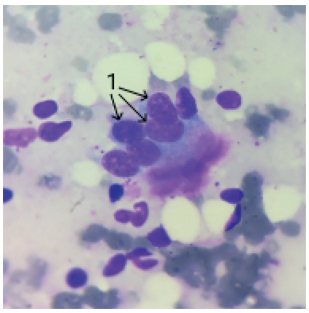

Figure 1. Cytogram of the lungs of the cadaver of a dog of the EG 2 immediately after death. Eyepiece 10×lens 100, stained according to Pappenheim-Kryukov
Note: *1 – ciliated epithelium*

Brain cytograms obtained immediately after the death of dogs of both research groups determine the average size of population cells: mainly neurons, oligodendrocytes, astrocytes. Neurons are large cells located singly. Their cytoplasm is blue with clear edges, without noticeable inclusions. The nucleus contains fine-grained chromatin and a single nucleolus.

The cytoarchitecture of neurons consists of a body (Fig. 3), from which an axon (Fig. 3) and a dendrite depart. The background of cytopreparations is represented by diffusely located red blood cells. Astrocytes are medium-sized cells placed discretely. Their cytoplasm is light blue, without noticeable inclusions, contains a single nucleus with fine-grained chromatin without a nucleolus.

Oligodendrocytes are smaller cells located discretely. Their cytoplasm is light blue and contains a single nucleus with fine-grained chromatin without nucleoli. Inclusions are not visualized. The background of the cytopreparation is vacuolized, represented by ordinary neuropil and partially neuronal processes of the brain (Fig. 4), light pink or light purple.

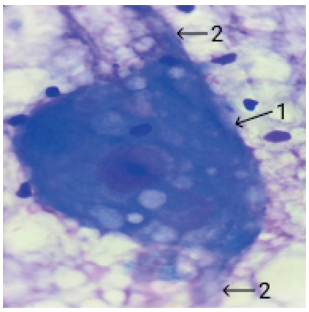

Figure 3. Cytogram of the brain of the cadaver of a dog of the EG 1 immediately after death. Eyepiece 10×lens 100, stained according to Pappenheim-Kryukov
Note: *1 – the body of the neuron; 2 – the axons of the neuron*

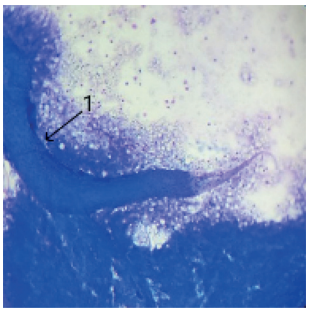

Figure 4. Cytogram of the brain of the corpse of a dog of the EG 2 immediately after death. Eyepiece 10×lens 100, stained according to Pappenheim-Kryukov
Note: *1 – accumulation of neuronal processes*

Liver cytograms obtained immediately after the death of dogs of both experimental groups reveal a dense population of hepatocytes of various shapes (Figs. 5, 6), mainly in the form of spheres, with light blue granular cytoplasm, a large part of them contains a single nucleus with crystalline structures (Figs. 5; 6) and nucleoli with compact chromatin.

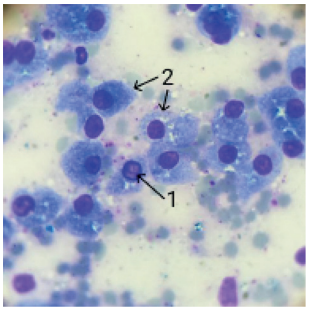

Figure 5. Cytogram of the liver of the cadaver of a dog of EG 1 immediately after death. Eyepiece 10×lens 100, stained according to Pappenheim-Kryukov
Note: *1 – crystal structures in the nucleus of a hepatocyte; 2 – hepatocytes*

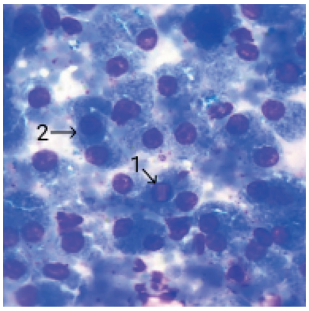

Figure 6. Cytogram of the liver of the cadaver of a dog of EG 2 immediately after death. Eyepiece 10×lens 100, stained according to Pappenheim-Kryukov
Note: *1 – crystal structures in the nucleus of a hepatocyte; 2 – hepatocytes*

Liver cytograms obtained from the cadavers of dogs of both experimental groups 3 hours after death do not substantially differ in morphological changes in hepatocytes (Figs. 7, 8) from the structure of necroptates for the previous study period. There are binuclear cells of the liver parenchyma (Fig. 7) of various stages of mitosis. Hepatocyte nuclei often contain separate crystal structures (Figs. 7, 8), which is common in the cytoarchitecture of liver cells in dogs of this age.

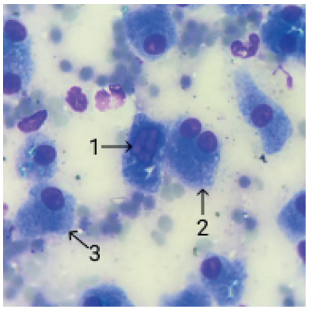

Figure 7. Cytogram of the liver of the corpse of a dog of the EG 1, 3 hours after death. Eyepiece 10×lens 100, stained according to Pappenheim-Kryukov
Note: *1 – crystal structures in the nucleus of a hepatocyte; 2 – binuclear hepatocytes; 3 – hepatocyte*

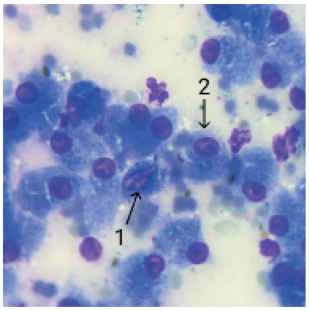

Figure 8. Cytogram of the liver of the corpse of a dog of the EG 2, 3 hours after death. Eyepiece 10×lens 100, stained according to Pappenheim-Kryukov
Note: *1 – crystal structures in the nucleus of the hepatocyte; 2 – hepatocyte*

On the examination micropreparation of the liver obtained from the cadaver of a dog from the EG 2, a background represented by white blood cells of a small diameter is visualized (Fig. 9). However, pancreatocytes and erythrocytes of high density are observed on the examination micropreparation of the pancreas of the cadaver of a dog from the EG 1 (Fig. 10).

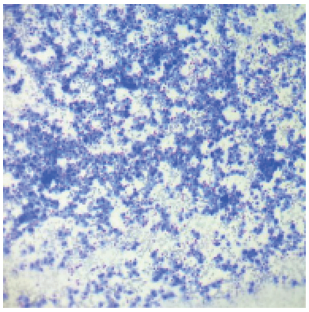

Figure 9. Cytogram of the liver of a dog cadaver of the EG 2, total cytogram 3 hours after death. Eyepiece 10×lens 10, stained according to Pappenheim-Kryukov

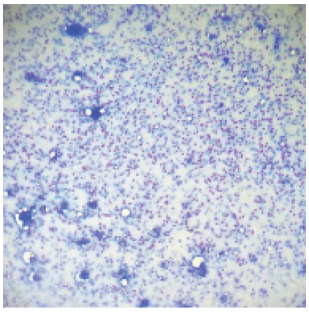

Figure 10. Cytogram of the pancreas of the corpse of a dog of the EG 1, total cytogram 3 hours after death. Eyepiece 10×lens 10, stained according to Pappenheim-Kryukov

Cells of the pancreas of cadavers of dogs of both experimental groups are mainly represented by the epithelium of exocrine ducts (Fig. 11) both in clusters without clear cytoarchitecture and in structured formations in the form of clusters of acinar and tubular forms (Fig. 11). The cells are polygonal, with a large volume of cytoplasm, dark blue, and with a small nucleus. Cytograms of the pancreas of the cadaver of a dog from the EG 1 show a cluster of stromal cells (Fig. 12) around the exocrine epithelium (Fig. 12) against a light blue background.

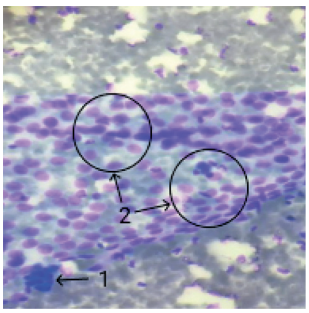

Figure 11. Cytogram of the pancreas of the cadaver of a dog of the EG 1, 3 hours after death. Eyepiece 10×lens 100, stained according to Pappenheim-Kryukov
Note: *1 – accumulation of exocrine epithelium; 2 – accumulation of exocrine epithelium in the form of tubular structures*

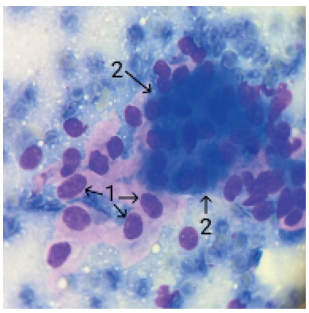

Figure 12. Cytogram of the pancreas of the cadaver of a dog of the EG 1, 3 hours after death. Eyepiece 10×lens 100, stained according to Pappenheim-Kryukov
Note: *1 – stromal cells; 2 – exocrine epithelial cells*

Acinar clusters of pancreatic cells (Fig. 13) of the cadaver of a dog of EG 2, 3 hours after death, polygonal with an eccentrically located round nucleus in each cell and two small nucleoli. Cytoplasm of exocrine epithelial cells in the form of a tubular structure (Fig. 14) occupies most of the area of each cell. The nuclei of individual pancreatic cells are basilar, uniform in structure, their shape is mainly spherical – from rounded to oval. Nuclear chromatin is speckled, with one small nucleoli with clear edges.

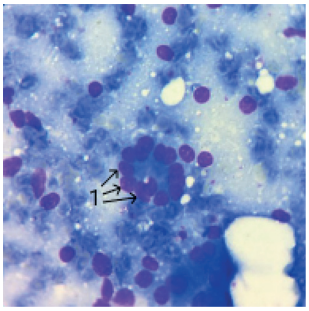

Figure 13. Cytogram of the pancreas of the cadaver of a dog of the EG 2, 3 hours after death. Eyepiece 10×lens 100, stained according to Pappenheim-Kryukov
Note: *1 – accumulation of exocrine epithelial cells in the form of an acinar structure*

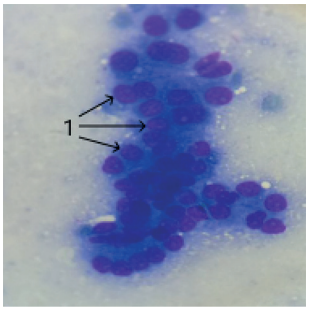

Figure 14. Cytogram of the pancreas of the cadaver of a dog of the EG 2, 3 hours after death. Eyepiece 10×lens 100, stained according to Pappenheim-Kryukov
Note: *1 – exocrine epithelial cells in the form of a tubular structure*

Cytograms obtained from smears-imprints of the kidneys of the cadavers of dogs of the EG 1, 6 hours after death contain, in large quantities, erythrocytes and renal epithelium of nephrons (Fig. 15). However, there are no cells with signs of structural degeneration. Examining the cytograms of the kidneys obtained from the cadavers of dogs of the EG 2, it was observed that the cells are mainly located discretely and in tubular clusters of renal epitheliocytes of nephrons (Fig. 16). The cytoplasm of kidney epithelial cells occupies half the cell area, most of them do not have a clear border between the cytoplasmic organelles. The colour of the cytoplasm varies from light blue to light pink. One nucleus with fine-grained chromatin is differentiated, and the nucleolus is not visible in it. Common features of the cytograms of the kidneys of cadavers of dogs of both experimental groups are numerous renal corpuscles and a protein-lipid, grey body that contains single unchanged neutrophils.

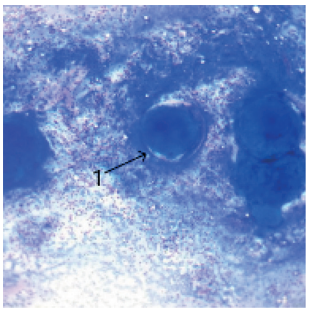

Figure 15. Cytogram of the kidney of the corpse of a dog of the EG 1, 6 hours after death. Eyepiece 10 × lens 10, stained according to Pappenheim-Kryukov
Note: *1 – renal body*

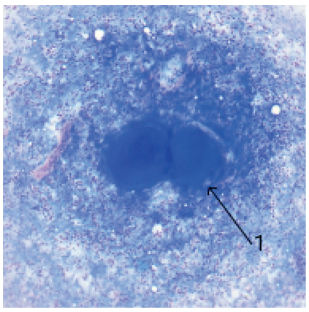

Figure 16. Cytogram of the kidney of the corpse of a dog of the EG 2, 6 hours after death. Eyepiece 10 × lens 10, stained according to Pappenheim-Kryukov
Note: *1 – renal body*

The cells of cytograms of the heart, obtained from the cadavers of dogs of the EG 1, 6 hours after death, are represented by fragmented cardiomyocytes (Fig. 17) in clusters. Their cytoplasm is dark blue with well-defined transverse striations (Fig. 18).

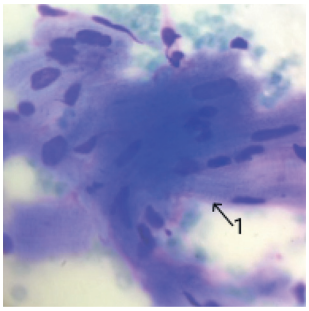

Figure 17. Cytogram of the heart of a cadaver dog of EG 1, 6 hours after death. Eyepiece 10×lens 100, stained according to Pappenheim-Kryukov
Note: *1 – cardiomyocytes*

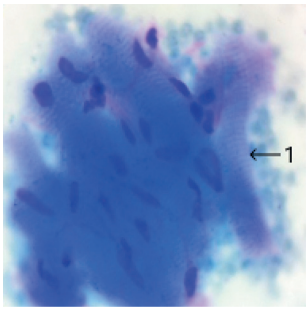

Figure 18. Cytogram of the heart of a cadaver dog of EG 1, 6 hours after death. Eyepiece 10×lens 100, stained according to Pappenheim-Kryukov
Note: *1 – cardiomyocytes*

Micropicture of cytograms obtained from the heart of cadavers of dogs of the EG 2, 6 hours after their death is characterized by a sparse cellular composition. The nuclei of cardiomyocytes (Fig. 19) are located parallel to the central axis of the cell, oval, without visible nucleoli. The background of cytopreparations is represented by red and white blood cells: red blood cells, neutrophils, small lymphocytes, and eosinophils (Fig. 20).

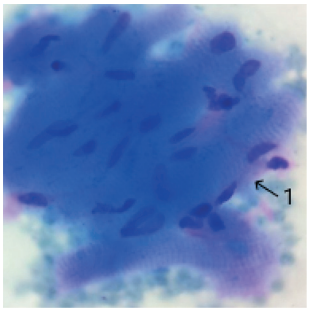

Figure 19. Cytogram of the heart of the cadaver of a dog of the EG 2, 6 hours after death. Eyepiece 10×lens 100, stained according to Pappenheim-Kryukov
Note: *1 – cardiomyocytes*

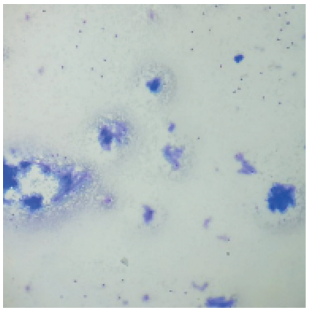

Figure 20. Cytogram of the heart of the cadaver of a dog of the EG 2, total cytogram 6 hours after death. Eyepiece 10 × lens 10, stained according to Pappenheim-Kryukov

Microscopic examination of smears-imprints of the spleen, obtained from the cadavers of dogs of the EG 1, 6 hours after their death, confirmed the presence of a heterogeneous population of white blood cells: lymphocytes of different sizes (Fig. 21), large lymphocytes (4%), small lymphocytes (79%), medium lymphocytes (11%), neutrophils (2%), eosinophils (1%), plasma cells (3%) (Fig. 21). No signs of cellular degeneration were recorded. However, when examining the cytological preparations of the spleen of the cadavers of dogs of group 2, 9 hours after their death, destroyed parenchyma cells (Fig. 22) and cocci-like bacteria (Fig. 22) were revealed for the first time. The background of cytograms is protein, grey, represented by high-density erythrocytes.

At 9 hours after the death of the dogs of both experimental groups, bacteria (Fig. 23, 24) and destroyed neurocytes (Fig. 24) were first detected in the brain.

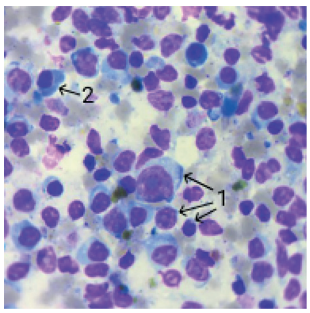

Figure 21. Cytogram of the spleen of the corpse of a dog of the EG 1, 6 hours after death. Eyepiece 10×lens 100, stained according to Pappenheim-Kryukov
Note: *1 – lymphocytes of varied sizes; 2 – plasmocide*

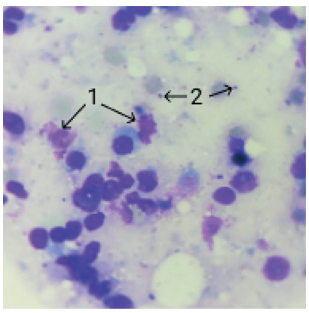

Figure 22. Cytogram of the spleen of the corpse of a dog of the EG 2, 9 hours after death. Eyepiece 10×lens 100, stained according to Pappenheim-Kryukov
Note: *1 – destroyed cells; 2 – bacteria*

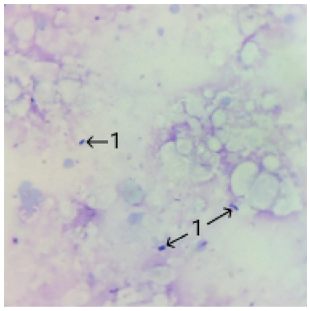

Figure 23. Cytogram of the brain of the cadaver of a dog of the EG 1, 9 hours after death. Eyepiece 10×lens 100, stained according to Pappenheim-Kryukov
Note: *1 – bacteria*

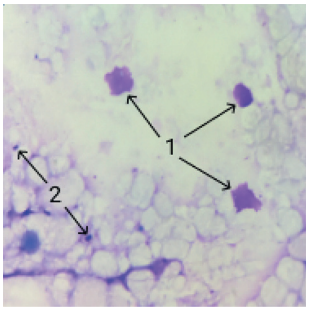

Figure 24. Cytogram of the brain of the corpse of a dog of the EG 2, 9 hours after death. Eyepiece 10 × lens 100, stained according to Pappenheim-Kryukov
Note: *1 – destroyed cells; 2 – bacteria*

The general cytogram of smears-prints of the myocardium of cadavers of dogs of both experimental groups is characterized by further fragmentation of cardiomyocytes (Fig. 25) with a violation of their striation (Fig. 26).

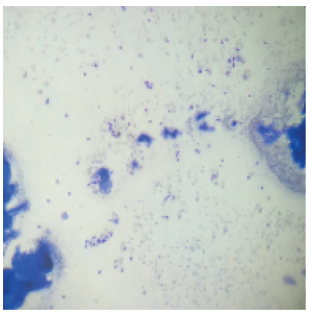

Figure 25. Cytogram of the heart of the cadaver of a dog of the EG 1, total cytogram 9 hours after death. Eyepiece 10×lens 10, stained according to Pappenheim-Kryukov

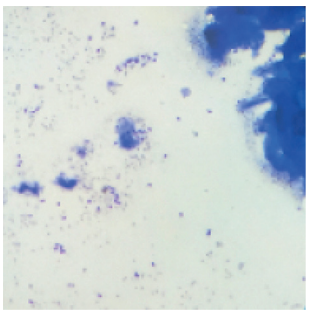

Figure 26. Cytogram of the heart of the cadaver of a dog of the EG 2, total cytogram 9 hours after death. Eyepiece 10×lens 10, stained according to Pappenheim-Kryukov

12 hours after the death of the dogs of both experimental groups, the number of bacteria in the brain increases. Bacteria are placed in clusters around neurocytes (Fig. 27). They are cocci and rod-shaped (Fig. 28). Due to the vital activity of bacteria, the destruction of brain substance cells increases (Fig. 28).

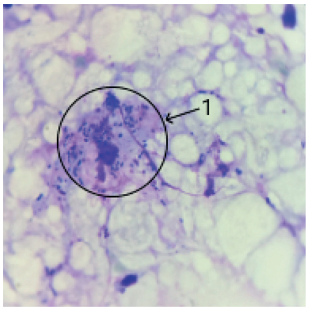

Figure 27. Cytogram of the brain of the cadaver of a dog of the EG 1, 12 hours after death. Eyepiece 10×lens 100, stained according to Pappenheim-Kryukovv
Note: *1 – accumulation of bacteria around the neuron body*

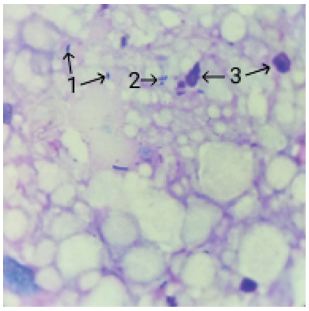

Figure 28. Cytogram of the brain of the cadaver of a dog of the EG 2, 12 hours after death. Eyepiece 10×lens 100, stained according to Pappenheim-Kryukov
Note: *1 – cocci-like bacteria; 2 – rod-shaped bacteria; 3 – destroyed neurocytes*

Intensive processes of cell destruction with simultaneous bacterial contamination were also observed in other internal organs. Thus, on the cytograms of the pancreas of the cadavers of dogs of EG 1, intensive bacterial colonization is noted (Fig. 29), and in the tissue of the spleen of cadavers of dogs of EG 2, apart from bacterial contamination (Fig. 30), significant destructive changes are detected (Fig. 30).

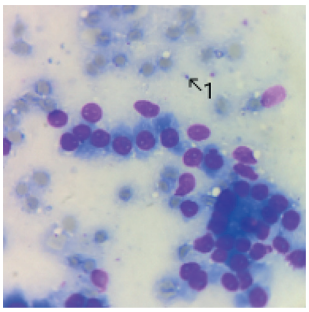

Figure 29. Cytogram of the pancreas of the corpse of a dog of the EG 1, 12 hours after death. Eyepiece 10×lens 100, stained according to Pappenheim-Kryukov
Note: *1 – bacteria*

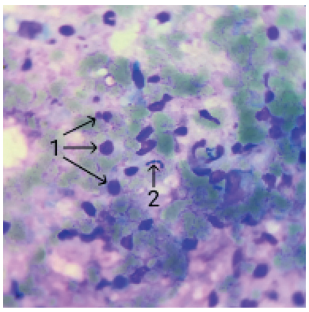

Figure 30. Cytogram of the spleen of the corpse of a dog of the EG 2, 12 hours after death. Eyepiece 10 × lens 100, stained according to Pappenheim-Kryukov
Note: *1 – destroyed cells; 2 – bacteria*

12 hours after death, it can be stated that on the cytograms of the liver obtained from the cadavers of dogs of both experimental groups, the processes of destruction of the parenchyma cells of the organ continue to intensify (Figs. 31, 32), which occurs against the background of colonization by bacteria (Figs. 31, 32).

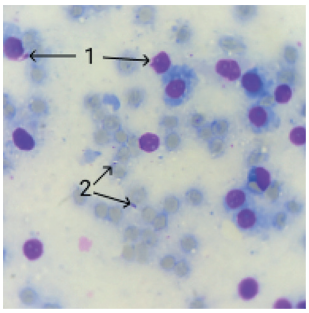

Figure 31. Cytogram of the liver of the corpse of a dog of the EG 1, 12 hours after death. Eyepiece 10×lens 100, stained according to Pappenheim-Kryukov
Note: *1 – destroyed cells; 2 – bacteria*

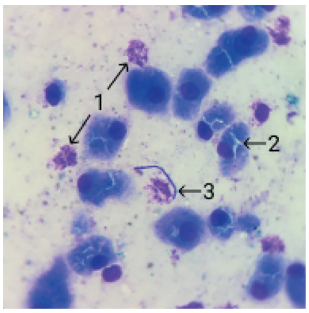

Figure 32. Cytogram of the liver of the cadaver of a dog of the EG 1, 15 hours after death. Eyepiece 10×lens 100, stained according to Pappenheim-Kryukov
Note: *1 – destroyed cells; 2 – hepatocyte; 3 – bacteria*

On the cytograms of the liver of the corpse of a dog of the EG 2, obtained 15 hours after its death, the destructive changes in the cells are noted (Fig. 33) and the number of bacteria (Fig. 33) do not differ significantly from the necroptates obtained in the previous periods of the study, except for the presence in the nuclei of hepatocytes of non-specific crystal structures (Fig. 33), which is a variant of the cytological norm for these animals in adulthood. 18 hours after the death of dogs of the EG 1, the presence of bacterial colonies around the bodies of neurons in the brain was noted (Fig. 34).

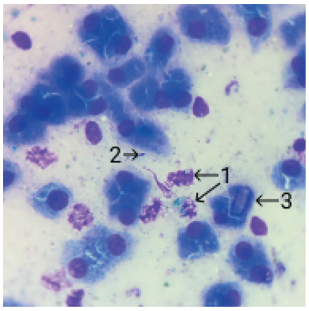

Figure 33. Cytogram of the liver of the corpse of a dog of the EG 2, 15 hours after death. Eyepiece 10×lens 100, stained according to Pappenheim-Kryukov
Note: *1 – destroyed cells; 2 – bacteria; 3 – crystal structure in the nucleus of the hepatocyte*

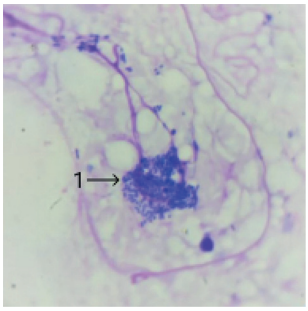

Figure 34. Cytogram of the brain of the cadaver of a dog of the EG 1, 18 hours after death. Eyepiece 10×lens 100, stained according to Pappenheim-Kryukov
Note: *1 – accumulation of bacteria around the neuron's body*

However, 21 hours after the death of dogs, bacterial contamination of the brain substance is detected in both experimental groups in the form of clusters of bacterial colonies (Figs. 35, 36) over the entire area of the object under study.

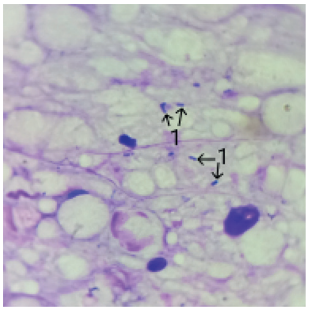

Figure 35. Cytogram of the brain of the cadaver of a dog of the EG 1, 21 hours after death. Eyepiece 10×lens 100, stained according to Pappenheim-Kryukov
Note: *1 – bacteria*

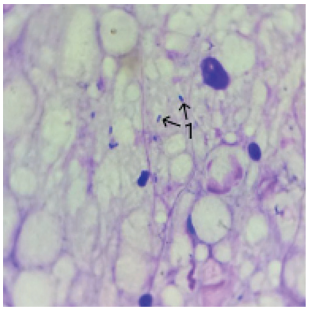

Figure 36. Cytogram of the brain of the cadaver of a dog of the EG 2, 21 hours after death. Eyepiece 10×lens 100, stained according to Pappenheim-Kryukov
Note: *1 – bacteria*

24 hours after the death of dogs, active destructive processes are noted in their spleen. Thus, on the cytograms of the spleens of the cadavers of dogs of both experimental groups, the prevalence of bacteria is observed (Figs. 37, 38) with localization over the entire area of cytopreparations. Thus, on cytological preparations, the processes of total destruction in the cellular composition were recorded (Figs. 37; 38).

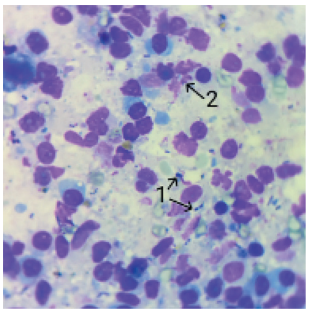

Figure 37. Cytogram of the spleen of the cadaver of a dog of the EG 1, 24 hours after death. Eyepiece 10×lens 100, stained according to Pappenheim-Kryukov
Note: *1 – bacteria; 2 – destroyed cells*

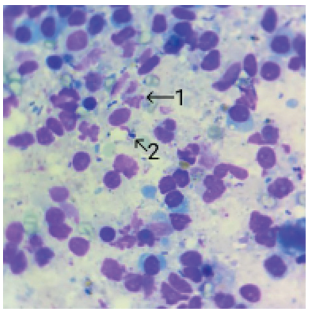

Figure 38. Cytogram of the spleen of the cadaver of a dog of the EG 2, 24 hours after death. Eyepiece 10×lens 100, stained according to Pappenheim-Kryukov
Note: *1 – destroyed cells; 2 – bacteria*

Attention is drawn to the cytological picture of the liver and heart, smears-prints from which were obtained a day after the death of animals. Necrobiotic processes in these internal organs are close to cytolysis. Only single indestructible hepatocytes are preserved on liver cytograms of cadavers of dogs of the EG 1 (Fig. 39). However, most cells are subject to total destruction (Fig. 39). At the same time, cytograms of the heart of cadavers of dogs of the EG 1 reflect fragmentation of cardiomyocytes (Fig. 40).

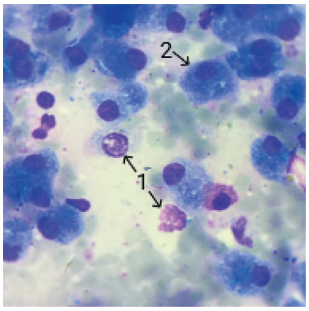

Figure 39. Cytogram of the liver of the cadaver of a dog of the EG 1, 24 hours after death. Eyepiece 10×lens 100, stained according to Pappenheim-Kryukov
Note: *1 – destroyed cells; 2 – hepatocyte*

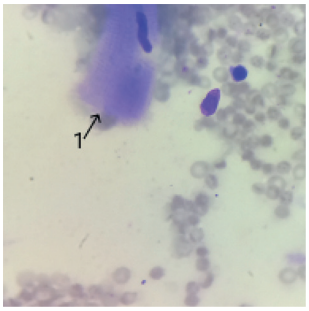

Figure 40. Cytogram of the heart of the cadaver of a dog of the EG 1, 24 hours after death. Eyepiece 10×lens 100, stained according to Pappenheim-Kryukov
Note: *1 – cardiomyocyte*

During the cytological examination of smears-imprints of internal organs after 12 hours of the experiment, their common optical property of eosinophilic staining was established, which is evidently associated with the beginning of the destruction of nuclear components of cells, lysis by karyolium, the exit of nuclear components outside the nucleus and their mixing with substance of the cytoplasm.

Observation of the quantitative dynamics of bacterial contamination of cadaver organs in dogs of both experimental groups at the same time intervals in the early post-mortem period allowed establishing that the intensity of its manifestation is specific for each of them (Table 1).

Table 1. Dynamics of bacterial contamination of internal organs of cadavers of dogs of experimental groups at the same time intervals of the early post-mortem period (n = 14)

Experiment interval (h)	Experimental group	Number of bacterial units on cytograms of internal organs						
		Lungs	Heart	Liver	Spleen	Kidneys	Pancreas	Brain
0-3	1	Bacterial contamination is not observed						
	2	Bacterial contamination is not observed						
3-6	1	–	–	–	1.36 ± 0.13	–	–	–
	2	–	–	–	1.83 ± 0.22	–	–	–
6-9	1	–	–	–	4.23 ± 0.06***	–	10.07 ± 0.11	1.01 ± 0.03
	2	–	–	–	4.51 ± 0.08***	–	11.83 ± 0.22^^^	1.34 ± 0.05^^
9-12	1	0.94 ± 0.04	–	–	8.23 ± 0.07***	2.97 ± 0.08^	16.07 ± 0.09***	1.49 ± 0.08**
	2	1.11 ± 0.03^	–	–	8.21 ± 0.13***	2.60 ± 0.09	13.86 ± 0.08****	1.99 ± 0.05***
12-15	1	1.13 ± 0.03**	–	1.11 ± 0.03	16.33 ± 0.12***	3.8 ± 0.04***	16.70 ± 0.06***	2.41 ± 0.05***
	2	1.16 ± 0.04	–	1.07 ± 0.05	15.70 ± 0.26***	3.51 ± 0.04****	16.16 ± 0.05***	3.24 ± 0.06***
15-18	1	1.23 ± 0.05	–	2.10 ± 0.06***	19.69 ± 0.36***	4.43 ± 0.08***	17.79 ± 0.07***	2.97 ± 0.08**
	2	1.29 ± 0.03*	–	2.61 ± 0.06***	18.86 ± 0.17***	4.51 ± 0.04***	18.33 ± 0.04***	3.93 ± 0.05***

Table 1, Continued

Experiment interval (h)	Experimental group	Number of bacterial units on cytograms of internal organs						
		Lungs	Heart	Liver	Spleen	Kidneys	Pancreas	Brain
18-21	1	1.33 ± 0.04	–	3.11 ± 0.08***	21.01 ± 0.21**	4.99 ± 0.05***	18.47 ± 0.04***	3.29 ± 0.04*
	2	1.37 ± 0.05	–	3.34 ± 0.07***	20.07 ± 0.06***	5.24 ± 0.06***	18.71 ± 0.04***	4.46 ± 0.06***
21-24	1	1.50 ± 0.03**	0.91 ± 0.05	3.97 ± 0.05***	22.54 ± 0.05***	6.86 ± 0.06***	19.20 ± 0.11***	4.43 ± 0.08***
	2	1.50 ± 0.02**	1.00 ± 0.05	4.26 ± 0.07$^{*\wedge}$	24.40 ± 0.22$^{***\wedge\wedge\wedge}$	7.53 ± 0.06$^{***\wedge\wedge\wedge}$	21.16 ± 0.39$^{***\wedge\wedge}$	5.77 ± 0.06$^{***\wedge\wedge\wedge}$

Note: *$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the indicators of the previous study period within the experimental group; $^{\wedge}P < 0.05$, $^{\wedge\wedge}P < 0.01$, $^{\wedge\wedge\wedge}P < 0.001$ compared to the indicators between the experimental groups in the same time frame of the experiment*

It was established that 3 hours after the death of dogs in both experimental groups, bacteria were not detected in any organ (Table 1). However, as early as 6 hours of follow-up, they are registered in the spleen of cadavers of dogs of these groups. During the experimental period, the number of bacteria in the internal organs of the cadavers of dogs of the experimental groups gradually increases. Thus, at 24 hours, it significantly increases by 16.57 times (P < 0.001) in the spleen of cadavers of dogs of the EG 1, and by 13.33 times (P < 0.001) – in cadavers of animals of the EG 2 compared to the period of their first detection (at 6 hours of the experiment). By comparing the quantitative indicators of bacterial contamination on the spleen cytograms of cadavers of dogs of the experimental groups 1 and 2, it was found that at 6 hours of examination, these indicators did not significantly differ from each other. At the same time, at 24 hours, the number of bacteria in the spleen of cadavers of dogs of the EG 2 was significantly higher by 1.08 times (P < 0.001) than in the spleen of cadavers of dogs of the EG 1.

The bacteria appeared in the pancreas and brain of cadavers of dogs in both experimental groups at 9 hours of examination. During the examination period, it was found that the number of bacteria gradually increased on the cytograms of the pancreas and brain of cadavers of dogs of the experimental groups 1 and 2.

Thus, at 24 hours, their number significantly increased by 1.9 times (P < 0.001) in the pancreas of the cadavers of dogs of the EG 1 and by 1.79 times (P < 0.001) – in the cadavers of animals of the EG 2 compared to the period when they were detected for the first time (at 9 hours of the experiment). Comparing the quantitative indicators of bacterial contamination on the cytograms of the pancreas of cadavers of dogs of the experimental groups 1 and 2 with each other, it was found that at 9 hours the number of bacteria in this organ of cadavers of dogs of the EG 2 significantly increased by 1.17 times (P < 0.001), and at 24 hours – by 1.1 times (P < 0.01) compared with such indicators in the EG 1.

An analogous trend was found when analysing brain cytograms in the cadavers of dogs of both experimental groups. Thus, at 24 hours of the experiment, the number of bacteria in the brain of cadavers of dogs of the EG 1 significantly increased by 4.39 times (P < 0.001) and in cadavers of animals of the EG 2 – by 4.31 times (P < 0.001) compared to the period when they were detected for the first time (at 9 hours of the experiment). Comparing the quantitative indicators of bacterial contamination on the brain cytograms of cadavers of dogs of experimental groups 1 and 2 with each other for 9 hours, it was found that the number of bacteria in this organ of cadavers of dogs of EG 2 significantly increased by 1.33 times (p < 0.01) than in EG 1. These dynamics persisted for 24 hours of the study. Thus, the number of bacteria in the brain of cadavers of dogs of EG 2 significantly increased by 1.3 times (P < 0.01) compared to EG 1.

After 12 hours of the experiment, the bacteria were recorded simultaneously in the lungs and kidneys of cadavers of dogs of both experimental groups. During the study period, the number of bacteria in the lungs and kidneys of cadavers of dogs in these groups gradually increased with varying intensity over the examination time intervals.

Thus, at 24 hours, the number of bacteria in the lungs of cadavers of dogs of the EG 1 was significantly higher by 1.6 times (P < 0.01) compared to the time when they were first detected. Comparing the indicators of bacterial contamination on the cytograms of the lungs of cadavers of dogs of experimental groups 1 and 2 with each other, it was found that by 12 hours, the number of bacteria in this organ of cadavers of dogs of EG 2 significantly increased by 1.18 times (P < 0.05) compared to EG 1. However, at 24 hours of examination, the values of bacterial contamination indicators did not significantly differ.

A comparable dynamic of bacterial contamination was found in the kidneys of cadavers of dogs of both experimental groups. Thus, at 24 hours in the kidneys of cadavers of dogs of EG 1, the number of bacteria is significantly higher by 2.31 times (P<0.001), and in EG 2 – by 2.9 times (P < 0.001) compared to the period when they are detected for the first time (at 12 hours of the experiment). Comparing the indicators of bacterial contamination on the kidney cytograms of cadavers of dogs of experimental groups 1 and 2 with each other, it was found that at 12 hours of observation, the number of bacteria in this organ of cadavers of dogs of EG 1 is significantly higher by 1.14 times (P < 0.05), and at 24 hours – by 1.1 times (P < 0.001) compared to EG 2.

According to the results of analysis of liver cytograms of cadavers of dogs of both experimental groups, it was found that bacteria in this organ appear for the first time at 15 hours of examination. Further, during the last study period, the number of bacteria in both groups gradually increases at certain examination time intervals. Thus, at 24 hours, the number of bacteria in the liver of cadavers of dogs of experimental groups 1 and 2 is significantly higher by 3.58 times (P < 0.001) and 3.98 (P < 0.05), respectively, compared to the period when they are detected for the first time (at 15 hours of the experiment). At the same time, at 24 hours, the number of bacteria in the liver of cadavers of dogs of EG 2 significantly increases by 1.07 times (P < 0.05) compared to EG 1. At 15 hours of the experiment, there was no statistically significant difference in the number of bacteria on the liver cytograms of cadavers of dogs of experimental groups 1 and 2.

Late bacterial contamination is inherent in the myocardium. Bacteria on the cytograms of the heart of cadavers of dogs of both experimental groups were detected for the first time after death at 24 hours of the experiment. Comparing the indicators of contamination of the heart by bacteria in the cadavers of dogs of experimental 1 and 2 with each other, a significant difference between these indicators has not been established. Analysing the quantitative dynamics of destructive cells in the organs of cadavers of dogs of both experimental groups at the same time intervals of the early post-mortem period, it was found that the intensity of their manifestation is specific for the organ in which they are detected (Table 2).

Table 2. Dynamics of destructive cells in the internal organs of dog cadavers at the same time intervals in the early post-mortem period (n = 14)

Experiment interval(h)	Experimental group	Number of units of destructive cells on cytograms of internal organs						
		Lungs	Heart	Liver	Spleen	Kidneys	Pancreas	Brain
0-3	1	No destructive cells were observed						
	2	No destructive cells were observed						
3-6	1	–	–	–	–	–	2.56 ± 0.26	–
	2	–	–	–	–	–	2.49 ± 0.12	–
6-9	1	–	–	–	9.73 ± 0.27	–	9.86 ± 0.10***	0.87 ± 0.04
	2	–	–	–	10.76 ± 0.25^	–	7.70 ± 0.08***	0.94 ± 0.04
9-12	1	0.44 ± 0.06	–	–	14.87 ± 0.25***	1.91 ± 0.06^^	12.33 ± 0.14***	1.16 ± 0.04**
	2	0.40 ± 0.04	–	–	16.53 ± 0.09***	1.49 ± 0.08	11.53 ± 0.16***	1.23 ± 0.05**
12-15	1	0.87 ± 0.04**	–	2.14 ± 0.10	22.37 ± 0.14***	2.63 ± 0.04***	13.16 ± 0.04**	1.67 ± 0.04***
	2	1.01 ± 0.03***	–	2.17 ± 0.04	18.59 ± 0.12***	2.41 ± 0.05***	12.61 ± 0.07***	1.67 ± 0.05***
15-18	1	1.36 ± 0.05***	–	3.76 ± 0.08***	23.40 ± 0.54	3.01 ± 0.03***	13.53 ± 0.07**	3.21 ± 0.07***
	2	1.34 ± 0.05**	–	3.64 ± 0.04***	22.93 ± 0.06***	2.97 ± 0.08**	13.53 ± 0.04**	3.71 ± 0.07***
18-21	1	1.96 ± 0.06***	–	5.26 ± 0.08***	27.09 ± 0.55**	3.21 ± 0.03**	14.24 ± 0.07***	5.99 ± 0.03***
	2	2.06 ± 0.06***	–	5.50 ± 0.05***	27.93 ± 0.41***	3.29 ± 0.04*	14.79 ± 0.04***	5.54 ± 0.05***
21-24	1	2.51 ± 0.03***	–	8.50 ± 0.13***	36.74 ± 0.43***	4.06 ± 0.07***	15.71 ± 0.10***	6.71 ± 0.05***
	2	2.49 ± 0.04**	–	8.94 ± 0.09***^	38.54 ± 0.16***^^	4.43 ± 0.08***^	15.83 ± 0.08***	6.46 ± 0.08***^

Note: **P < 0.05, **P < 0.01, ***P < 0.001 compared to the indicators of the previous study period within the experimental group; ^P < 0.05, ^^P < 0.01, ^^^P < 0.001 compared to the indicators between the experimental groups in the same time frame of the experiment*

It was established that 3 hours after the death of dogs of both experimental groups, destructive cells were not detected in any organ (Table 2). However, as early as 6 hours of the experiment, destructive cells are registered in the pancreas of cadavers of dogs of both experimental groups, although a statistically significant difference in their number has not been established.

During the study period, the number of destructively altered cells in the studied organs of dog cadavers gradually increases. Thus, at 24 hours, their number increases significantly by 6.14 times (P < 0.001) in the pancreas of cadavers of dogs of EG 1 and by 7.71 times (P < 0.001) – by EG 2 compared to the period when they are detected for the first time (at 6 hours of the experiment).

At 9 hours of the experiment, destructively altered cells were found in the spleen and brain of cadavers of dogs of both experimental groups. During the entire study period, the number of destructive cells in the spleen and brain of dog cadavers in both experimental groups gradually increased. Thus, at 24 hours of the experiment, their number in the spleen of cadavers of dogs of EG 1 significantly increases by 3.78 times (P < 0.001) and by 3.58 times (P < 0.001) – EG 2 compared to the period when they are detected for the first time (at 9 hours of the experiment). Comparing the number of destructive cells on the spleen cytograms of cadavers of dogs of experimental groups 1 and 2 with each other at 9 hours of the experiment, it was found that the number of destructive cells in this organ of cadavers of dogs of EG 2 is significantly greater by 1.14 times (P < 0.05) compared to that in EG 1. Similar dynamics were found at 24 hours of the experiment, when the indicator of the number of destroyed cells in the spleen of cadavers of dogs of EG 2 significantly increased by 1.05 times (P < 0.01) compared to its values in EG 1.

During the analysis of brain cytograms of cadavers of dogs of both experimental groups, it was found that at 24 hours of the experiment, the number of destructive cells in this organ of cadavers of dogs of experimental groups 1 and 2 is significantly higher by 7.71 times (P < 0.001) and 6.87 times (P < 0.001), respectively, compared to the period when they are detected for the first time (at 9 hours of the experiment). There was no statistically significant difference in the number of destructive cells on the brain cytograms of the cadavers of dogs of experimental groups 1 and 2 at 9 hours of the experiment. However, at 24 hours of the experiment, the number of destructive cells in the brain of cadavers of dogs of EG 1 significantly increased by 1.04 times (P<0.05) compared to that of cadavers of dogs of EG 2. The obtained data on the post-mortem cytomorphology of brain cells coincide with the results of the studies of Wunsche *et al*. (2016), who investigated the cytograms of the human brain to identify lifelong organic lesions of its membranes during septic processes.

After 12 hours of the experiment, destructive cells appear in the lungs and kidneys of cadavers of dogs of both experimental groups.

Thus, during the study of cytograms of the lungs of cadavers of dogs of experimental groups 1 and 2, it was found that the number of destructively altered cells significantly increases by 5.7 times (P < 0.001) and 6.23 times (P < 0.01),

respectively, compared to the period when they are detected for the first time (at 12 hours of the experiment). Comparing the number of destructive cells on the cytograms of the lungs of cadavers of dogs of experimental groups 1 and 2 at 15 and 24 hours of the experiment, no statistically significant difference was established.

In the kidneys of cadavers of dogs of EG 1, the number of destructive cells at 24 hours was significantly greater by 2.13 times ($P < 0.001$) and 2.97 times ($P < 0.001$), respectively, compared to the period when they were detected for the first time (at 12 hours of the experiment). At the same time, at 12 h of the experiment, the number of destructive cells in the kidneys of the cadavers of dogs of EG 1 is significantly 1.25 times greater ($P < 0.01$) compared to those of cadavers of dogs of EG 2. However, at 24 hours, this indicator in the kidneys of cadavers of dogs of EG 2 significantly increased by 1.1 times ($P < 0.05$) than in cadavers of dogs of EG 1.

Examining the cytograms of the livers of dog cadavers in both groups, it was established that destructive cells in this organ appear for the first time at 15 hours of observation and their number gradually increases during the experimental period. Thus, at 24 hours of the experiment, the number of destructive cells in the liver of the cadavers of dogs of experimental groups 1 and 2 probably increased by 3.97 times ($P < 0.001$) and 4.11 times ($P < 0.001$), respectively, compared to the time when they were first detected (at 15 hours of the experiment). At 24 hours of the experiment, the number of such cells in the liver of cadavers of dogs of EG 2 significantly increased by 1.05 times ($P < 0.05$) than in cadavers of dogs of EG 1 (Table 2).

Comparing the number of destructive cells on liver cytograms of dog cadavers between experimental groups 1 and 2 at 15 hours of the experiment, no statistically significant difference was found. The obtained data completely coincide with the studies of Ceciliason *et al*. (2021), who established the sequence of processes of necrobiosis of liver cells in humans.

During the study of cytograms of the heart of cadavers of animals of both experimental groups, no destructive changes in cells were registered during the experimental period. The obtained data on the cytomorphology of the heart confirm the results of the studies of Welson *et al*. (2021), who prove that the processes of fragmentation and vacuolization of the cytoplasm in rat cardiomyocytes begin no earlier than 96 h after the death of the animals.

Thus, summarizing the results obtained during the cytomorphological study of the compact organs of dog corpses with different indicators of mass and fatness, the authors of this study believe that the most intense cadaveric dynamics of bacterial contamination and cell destruction in the first day of the post-mortem period occurs in the spleen and pancreas. Therewith, pronounced putrefactive biotransformation of the spleen is caused by decomposition products in these organs. Necrobiotic changes that develop intensively in the cells of the spleen and pancreas evidently provoke bacterial contamination. At the same time, bacterial contamination of pancreatic cells, which was observed later than in the spleen, may also be the result of an inhibitory effect on the intensity of bacterial growth of autolytic enzymes of exocrine epithelial cells. Therefore, the intensity of dynamics of cytomorphological changes in spleen and pancreatic cells is of informative value when deciding on the duration of the post-mortem period during the first day after the death of dogs.

Conclusions

Based on a randomized study of cells of compact organs of cadavers of dogs, it was proved that only cytomorphological changes in spleen and pancreas cells in terms of their intensity have expert information content to solve the question of the prescription of death during the first day of the post-mortem period in dogs, regardless of their body weight and fatness. The dynamics of bacterial contamination and destruction of brain, kidney, and lung cells are moderately informative, while the liver and heart are uninformative.

The most intense simultaneous dynamics of bacterial contamination and cell destruction occurs in the spleen and pancreas of dog cadavers, regardless of their body weight and fatness, which is evidently associated with autolysis of pancreacites, the release of a considerable number of proteolytic enzymes from their lysosomes. Simultaneous pronounced putrefactive biotransformation of the spleen is caused by the emergence of a considerable number of protein catabolism products. Necrobiotic changes that dynamically develop in the cells of the spleen and pancreas obviously initiate intense bacterial contamination. Bacterial contamination of pancreatic cells, which was observed later than in the spleen, is also explained by the inhibitory effect of autolytic enzymes of exocrine pancreatic epithelial cells on the intensity of bacterial growth.

The dynamics of destructive post-mortem processes in the cells of internal organs of dog cadavers with different weights and fatness during the experimental period does not significantly differ and develops with the same intensity. However, apart from the direct dynamics of increasing the indicators under study, there is a paradoxical dynamics of cell destruction in the kidneys, pancreas, and brain of dog cadavers and bacterial contamination of the liver and kidneys. In the future, it is planned to continue investigating other cytomorphological characteristics of necroptates of internal organs of dogs in the early post-mortem period in the aspect of forensic veterinary examination.

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Цитоморфологічна характеристика некроптатів внутрішніх органів собак у ранньому постмортальному періоді в аспекті судово-ветеринарної експертизи

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Анотація. Актуальність дослідження зумовлена потребою судово-ветеринарної експертизи трупів тварин щодо наукового обґрунтування інформативних діагностичних критеріїв для оцінки давності настання смерті, особливо у ранньому постмортальному періоді. Проте, інформація щодо ранніх постмортальних змін трупів собак на мікроструктурному рівні в українській та зарубіжній науковій літературі має досить фрагментарний характер. У зв'язку з цим дана стаття спрямована на з'ясування інформативності динаміки процесів клітинної деструкції та бактеріальної контамінації внутрішніх органів трупів собак упродовж першої постмортальної доби для встановлення вірогідних експертних критеріїв давності настання смерті підекспертних тварин під час проведення судово-ветеринарної експертизи. Провідним підходом до дослідження цієї проблеми є методи отримання від трупів собак серії некроптатів із легенів, серця, печінки, селезінки, нирок, підшлункової залози та головного мозку, через однаковий часовий інтервал упродовж першої доби після настання смерті. У цитологічних препаратах, отриманих з некроптатів, підраховували кількість зруйнованих клітин і бактеріальних одиниць за допомогою оптичної мікроскопії. За результатами динаміки бактеріальної контамінації та інтенсивності морфологічних змін клітин селезінки та підшлункової залози встановлена їхня експертна інформативність щодо вирішення питання про давність настання смерті собак, незалежно від показників ваги та вгодованості. З'ясовано, що динаміка бактеріальної контамінації і клітинної деструкції головного мозку, нирок і легенів трупів собак мають середню експертну інформативність, а печінки та серця – не інформативні. Доведено, що динаміка руйнівних післясмертних процесів у клітинах компактних органів трупів собак різної маси та вгодованості на відповідних термінах вірогідно не відрізняються та розвиваються з однаковою інтенсивністю. Отримані результати дослідження матимуть значення як у теорії судово-ветеринарної експертизи, так і безпосередньо прикладне, зокрема, під час вирішення судовим експертом питання щодо давності настання смерті тварини

Ключові слова: підекспертні тварини, трупна біотрансформація, клітинна деструкція, бактеріальна контамінація, посмертні зміни органів

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