

Experimental study of tropism of cultivated canine parvovirus in the immunogenesis organs of puppies

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The immune system unites the organs and the tissues that protect the organism against genetically alien cells or substances entering the organism from the environment. Canine parvovirus is an etiologic agent of hemorrhagic gastroenteritis and causes a significant problem for veterinary medicine due to high level of morbidity and mortality, mostly among dogs, because of fast progression without immune-complement response. In this study, based on the results of our clinical, virological, histological, histochemical and morphological assays, we determined the pathogenetic role of parvovirus in sick dogs experimentally infected per os, specifically with isolated canine parvovirus (Antaeus) with titer of infectious activity equaling $3.80 \pm 0.008 \lg \text{TCID}_{50}/\text{cm}$, cultivated on heterologous cell cultures. This allowed us to clarify, add to and generalize the data on the pathogenesis of the disease and determine pathohistological and histochemical changes in the immunogenesis, since the studied virus expresses immune-suppressive properties, leading to ruination of the locomotor ability of the organism and fast lethal outcome. The study of pathomorphological changes was carried out using pathoanatomical and histologic methods. Pathoanatomical material from the autopsy of puppies aged 45 days was fixed in 10% aqueous solution of neutral formaline and embedded in paraffin. Having parvoviral infection, dogs experience pathomorphologic changes in immune-complement organs, indicating inhibition of the immunogenesis function during an infectious disease of viral etiology. In the immunogenesis organs of puppies with the experimental reconstruction of parvoviral enteritis, we microscopically determined the following: edema of the cortex and medulla, disorganization of thymic corpuscles, and impairment of processes of differentiation of lymphocytes in the cortex and medulla of the thymic lobules; edema and large areas of accumulation of hemosiderin in the spleen as a result of breakdown of large amount of erythrocytes; acute inflammatory hyperemia of the parenchyma, swelling of sinuses, serous and serous-hemorrhagic lymphadenitis in lymph nodes. The complex of histologic changes in the immune protection organs, which we found in the conditions of experimental reconstruction of parvoviral infection, can be considered a distinct criterion for pathomorphologic differentiation diagnostics of parvoviral enteritis in dogs.

Keywords: parvovirus; immune organs; spleen; thymus; lymph nodes; pathoanatomical dissection; pathomorphology.

Introduction

The immune system is one of the most perfect mechanisms of living organisms. Over the recent decades, the influence of immunology, integrating organs on other biological, biochemical and morphological sciences has reached the dominating level (Kisera et al., 2019; Martyshuk et al., 2020). Other than functions of protective mechanism against infections and tumours, immunology is of greatest significance in cases of allergic diseases, autoimmune processes, transplantation, particularly blood transfusion, or even aging of an organism. The immune system is formed by a complex of organs and tissues, which provide protection against alien endo- and exogenic effects (Khariv et al., 2017; Gerardo et al., 2020). During infectious diseases, antigen factors cause morphological changes in the immune system organs, namely inflammatory processes (non-specific) and immunomorphological (specific) changes (Kvaratskhe-liya et al., 2016; Pereira et al., 2019; Radzykhovskiy et al., 2020).

The issue of combating infectious diseases of animals is still the most relevant for modern veterinary medicine, especially viral pathology (Ohshima & Mochizuki, 2009; Venn et al., 2017), characterized by a broad

range of spread with long-term impact on animals' health, specifically high lability of clinical signs, which complicate the diagnosis for a practicing veterinary doctor (Borland et al., 2020; Moore et al., 2021).

One of the commonest highly contagious and essential enteropathogens in dogs is parvovirus, which infects dogs all around the globe, causing parvoviral enteritis, one of the main causes of morbidity and mortality of puppies (Summa et al., 2012; Sato-Takada et al., 2022). Death of dogs caused by parvoviral enteritis ranges 10% to 70%, depending on form of disease manifestation.

Pathogenic impact starts from tonsils and lymph nodes in the pharynx and neck, lymph cell of Peyer's patches of the intestine, evolving to the system-wide form by spreading across the organism with blood flow, or in plasma, or infected lymphocytes (Nandi & Kumar 2010). Also, the virus can start to develop from the mesentery lymph nodes and thymus. Parvovirus has immune-depressing effect – damages immune cells, namely lymphocytes, where active division stage and increase in own infected cells occur, thus being “invisible” for other macrophages. Then, “altered lymphocytes” migrate to the blood flow. Most infected lymphocytes die as a result, causing decrease in the number of circulating lym-

phocytes and emergence of lymphopenia (Geetha, 2015; Mylonakis et al., 2016) and even necrosis of lymphoid tissue in lymph nodes and the virus begins to intensely reproduce, starting already from the second day, reaching maximal titer on the 4th day (Kilian et al., 2018; Mazzaferro, 2020).

Over the recent years, five different antigen variants of parvovirus have emerged around the globe, which is dangerous for many animals, and new variants express pathogenicity also toward felines. Despite presence of highly specific diagnostics and effective vaccines, outbreaks of parvovirus have been recorded all around the world, characterized by novel antigen variants and broadening of the range of susceptible animals and low efficacy of vaccination (Canuti et al., 2022; Singh et al., 2022). Parvoviruses (from Latin *parvus* – small) comprise a group of pathogenic viruses which poses a threat to both vertebrates and insects. The virion of parvoviruses has relatively simple structure and is composed of only three proteins and linear single-chain DNA molecule (Kalinina, 2016; Miranda & Thompson 2016).

Parvoviral enteritis of canines was recorded for the first time in Belgium in 1976, though some authors report the first appearance in 1978 in North America, but despite more than forty years of studies, the issue of integrated study of parvoviral infection is currently relevant not only for Ukraine (Mira et al., 2018; Milićević et al., 2022; Silva et al., 2022).

Manifestation of parvoviral infection had several clearly expressed peaks of activity, and the beginning of which reached the scales of pandemics in the 1980s and has been at a significant level up to the 1990s. The next, less expressed but more characteristic peak of activity was recorded in the 1990s and the third, less pronounced peak occurred in the early 2000s (Agnihotri et al., 2017; Santana et al., 2022).

During the analysis of the literature sources and performance of epizootologic studies, we noted significant spread of the parvoviral infection in Ukraine, and to a certain degree, often among immunized animals with high percentage of mixed infection (Caddy, 2018; Raza et al., 2018), making specific manifestation of clinical and pathomorphologic signs impossible. In the literature sources that were available to us, the problematic of immune response of the organism to the action of viral agent was usually described at the hematological level, whereas the pathogenic effect of antigen on immune-competent organs is usually described for productive animals, and very briefly for canines (Lisova, 2011; Kotsymbas & Shkil, 2018).

To summarize the aforesaid, we state that parvovirus displays immune-depressive action – damages immune cells and is the main intestinal virus of carnivore animals all around the world, which poses a serious threat to dogs. To this day, the issue of pathomorphology of this disease exists because broad immunization of animals alters its clinical-morphological picture. Moreover, the immune-morphological aspects of the disease's pathogenesis are practically unexplored. Therefore, the diagnostics of the parvoviral infections still remains imperfect. Taking into account the relevance of this issue, the results of our studies are elaboration, addition to and generalization of the data on pathomorphology of the immunogenesis organs of dogs in the conditions of experimental reconstruction of parvoviral infection, which give opportunities for more detailed studies of the effect of the disease pathogen on the organism of the animal suffering this pathology.

Materials and methods

During the studies, we followed the main rules of the Good Laboratory Practice GLP (1981), positions of the “General Ethical Principles of Experiments on Animals”, approved by the First National Congress of Bioethics (Kyiv, 2001). The entire experimental part of the studies was conducted according to the requirements of the principles of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Strasbourg, 1986), “Rules of Performing Studies using Experimental Animals”, adopted by the Order issued by the Ministry of Healthcare of Ukraine No. 281 as of November 1, 2000 “On Measures for Further Improvement of Organization Forms of Work using Experimental Animals” and according to the Law of Ukraine “On Protection of Animals from Cruel Treatment” (No. 3447-IV as of 02/21/2006, Kyiv). In the experiment, we used 5 dogs aged 45 days, crosses between Labrador and mongrel dog. The animals were in satisfac-

tory condition with no signs of diseases or pathology. To exclude the impact of parasitic agents, on the 21st and 34th day since birth of dogs, we carried out dehelminthization using Pirantel anthelmintic. The puppies were kept in separate boxes in an infectious isolator, were tested for absence of infection with IXA (test strip) using diagnostic VetExpert test system (CAV Ag, CDV Ag, CPV/CCV Ag and Rota Ag).

After the acclimation and adaptation of puppies weighing 2.95 ± 0.107 kg, they were divided into two groups, the first comprising animals that were infected with cultured parvovirus, and the second being the control group for detecting spontaneous emergence of disease in case of infection prior to the experiment. To infect, we used isolated and PLC-confirmed parvovirus obtained from a dead dog. Infected biological material was cultivated on heterologic cell cultures, particularly reinoculated lines of cultures of SPEV cells (kidneys of pig embryo), VNK-21 (kidneys of Syrian hamster), RK-13 (rabbit kidneys). In the experiment, we used cultivated viral isolate, which had 90–100% manifestation of CPE for 10 days. Virus-containing cultural isolate was chilled at the temperature of -24 °C. Prior to the use, it was reproduced at room temperature, and the mat with culture liquid was occasionally shaken by spontaneous movements for improvement of burst of cells and exit of the virus. The experimental animals were infected per os using the dose of 5 mL/kg of suspension of strain with titer of infection activity of 4.8 ± 0.04 lg of TCID₅₀/cm once a day. The clinical condition of every dog was controlled daily over 6 days. On days 4–5, the clinical symptoms were recorded (vomiting, diarrhea, loss of appetite, start of dehydration). On ethical grounds, we performed euthanasia.

In the experiment, we used the Antaeus parvovirus strain, isolated in Ukraine for the first time, stored in the Depository of the State Scientific-Control Institute of Biotechnology and Strains of Microorganisms during primary deposition (registration number given to the microorganism strain in the Depository is 734, as of 12/10/2018).

In the prosectorium of the Department of Anatomy and Histology of the Faculty of Veterinary Medicine, the animals were autopsied in supinate position, using the method of complete evisceration, in generally accepted order. The material for the study was fragments of the organs (spleen, thymus, somatic and visceral lymph nodes), which were dissected during pathoanatomic autopsy of puppies ($n = 5$), after humane euthanasia by sodium thiopental, in compliance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Decaro et al., 2008; Decaro et al., 2012; Mitchell et al., 2013; Nychyporuk et al., 2022).

In those cases, presence of parvovirus, without other associations, was confirmed by immune-enzymatic analysis (IEA) and immune-chromatographic analysis (ICA) during the analysis of fecal samples.

For histological study, freshly collected material was immediately fixed in 10% aqueous solution of neutral formalin, with 30 days fixation period with following stage-by-stage embedment into paraffin. Using a sledge microtome, we obtained 6–10 μ m thick histological sections. For histological identification of nucleic acids of proteins of glycosaminoglycans and Schiff iodine acid-positive substances, as fixating method, we used Carnoy's fluid. After deparaffinization, prepared histological sections were stained using Carazzi's hematoxylin and eosin according to the classic scheme. To determine the content and inner-cellular localization of DNA and RNA, the sections were stained using halocyanin-chrome alum according to the Einarson's technique. The basic and acidic proteins were detected using the Michel-Calvo technique, glycosaminoglycans – using alcian blue according to Steadman. Schiff iodine acid-positive substances were determined using Schiff iodine acid (SIA) according to McManus (Goralsky et al., 2017).

The general histological structure and microstructural changes in histological preparations were studied under an MC 100LED (Micros Austria) light microscope at 70 to 1,000 times magnification. Microphotographing of the histological preparations was done using a CAM V200 video camera, installed into a Micros MC 50 microscope.

Results

The first general symptoms of parvoviral infection were determined on the 4th day, whereas the beginning of specific clinical signs was seen

on the sixth day. In this period, we performed IXA diagnostics using a VetExpert test system for presence of antigen. During the experimental parvovirus, the clinical signs were depression, loss of appetite, fast loss of body weight of puppies and dehydration. We observed porridge-like feces with fragments of slime, and a day later we observed hemorrhage cords. Unclearly manifested vomiting was seen. To prevent the expression of pathogenic effect of indigenous microflora, we euthanized the animals.

During the pathoanatomical dissection of corpses of puppies that were experimentally infected with parvovirus isolate cultivated in heterologic cell culture, we observed that macroscopic changes somewhat differed from such in dogs that died of spontaneous course of this disease. Corpses of the puppies had low fat content. The skin and subcutaneous adipose tissue were dry, indicating dehydration of the organism. Noticeable mucous membranes were cyanotic.

As a result of pathoanatomical dissection, we determined that the spleen was of dark cherry colour, had weak consistency, was reduced in size, which caused a somewhat wrinkled capsule with dull margins. In the section, the organ's parenchyma did not protrude out of the cut capsule, the pattern of the tissue was smoothed, the pulp had fine-grained structure, and the scrape from the section's surface was insignificant. Notable macroscopic changes were found in the thymus. The organ had uneven hyperaemia, was of pink colour, permeated with serous substance, the stroma of the organ was in the condition of edema, causing some areas of the organ to notably increase and protrude above its general surface.

In all analyses of somatic and visceral lymph nodes that preserved lobular structure, we saw signs of serous-hemorrhagic lymphadenitis. They were enlarged, their capsule was tense, the parenchyma of the cortical zone had notably increased moisture, protruded from the section, and were unevenly pink on the surface and the sagittal section, having reddish tone in some places. After the length-wise cut, the halves were not connected.

Histological studies of the thymus revealed plethora of the vessels, such blood vessels of the parenchyma and the organ's stroma were notably enlarged. Between the thymic lobules, there was notable uneven edema of the connective-tissue stroma of the organ and expressed edema of the cortex and medulla in most of the thymic lobules (Fig. 1, 2). We determined disorganization of a large amount of thymic corpuscles. Its cells were in the condition of granular dystrophy. Single thymic corpuscles necrotized. Such changes may indicate impairment in maturation and differentiation of T lymphocytes in the thymus.

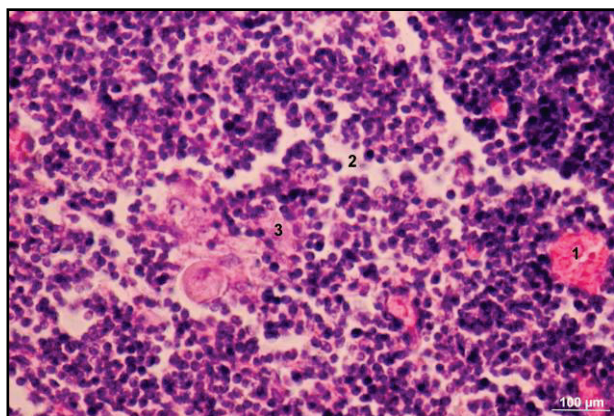


Fig. 1. Fragment of microscopic structure of the medulla of thymic lobule of an experimentally infected puppy: 1 – enlarged, plethorized blood vessel; 2 – edema of the parenchyma; 3 – disorganization of thymus corpuscule; hematoxylin and eosin

During the histological studies, we observed somatic and visceral lymph nodes having a varying pattern of microscopic changes. However, those changes were most notable in intestinal, hepatic lymph nodes and lymph nodes of pelvic cavity. Histologically, we observed disorder in the tissue structure, enlargement of sinuses, sharp inflammatory hyperemia of the parenchyma (Fig. 3–5). We determined serous and serous-hemorrhagic lymphadenitis.

Blood vessels of all somatic and visceral lymph nodes were notably enlarged, overfilled with blood. The cortex and medulla were swollen (Fig. 3–5). At the same time, in the medulla, we observed its diffusive

infiltration by a large number of erythrocytes (Fig. 5, 6), at the same time, in the cortex, we saw no such an infiltration (Fig. 3, 4).

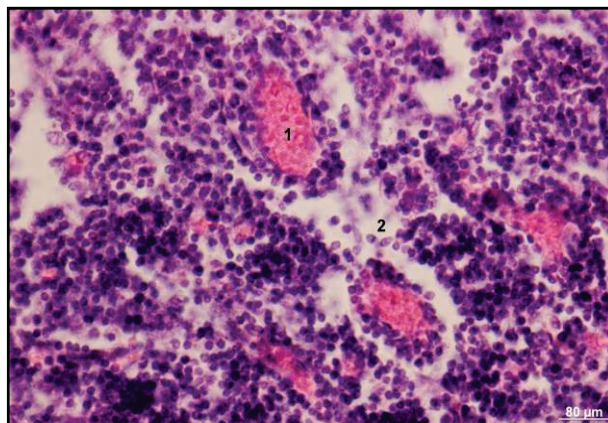


Fig. 2. Fragment of microscopic structure of the cortex of thymic lobule of experimentally infected puppy: 1 – enlarged blood vessel, overfilled with blood; 2 – edema of the parenchyma; hematoxylin and eosin

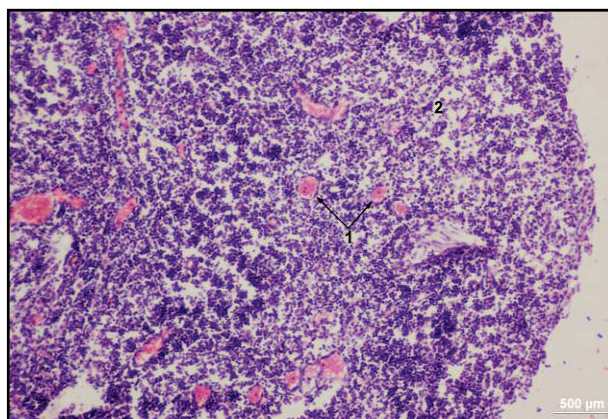


Fig. 3. Fragment of microscopic structure of the cortex of the intestinal lymph node of an experimentally infected puppy: 1 – enlarged blood vessels, overfilled with blood; 2 – edema; hematoxylin and eosin

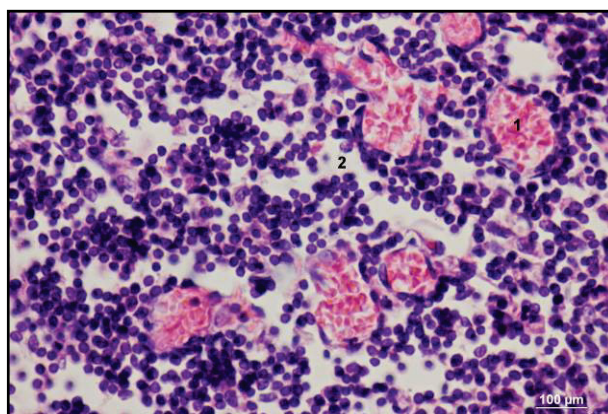


Fig. 4. Fragment of microscopic structure of the cortex of inguinal lymph node of an experimentally infected puppy: 1 – enlarged blood vessel, overfilled with blood; 2 – edema; hematoxylin and eosin

Lymph nodules were unexpressed, or completely absent (Fig. 3). In the cortical substance, we observed single macrophages, most of which had close contact with lymphocytes (Fig. 7).

In the medulla, there were also localized single macrophages that contacted lymphocytes, as well as erythrocytes of altered form (Fig. 8).

As a result of histological study of the spleen, we determined that its capsule was slightly swollen, which was seen only in great increases in the eyesight of the histological preparation. We found stagnant hyperemia, edema of the stroma and parenchyma, plethora of the vessels. Blood pulp

was swollen insignificantly (Fig. 9), and contained numerous sites of large amounts of erythrocytes.

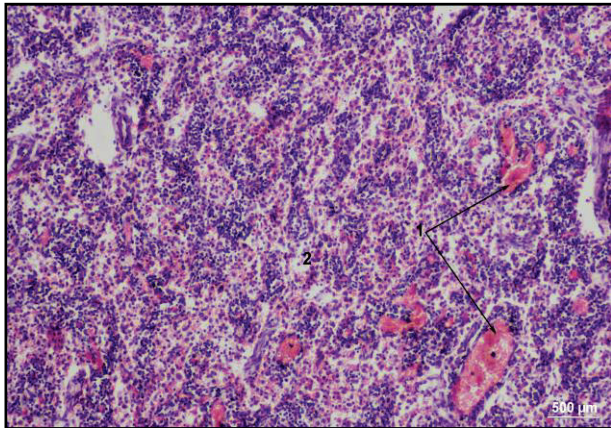


Fig. 5. Fragment of microscopic structure of the medulla of intestinal lymph node of an experimentally infected puppy: 1 – enlarged blood vessel, overfilled with blood; 2 – edema; hematoxylin and eosin

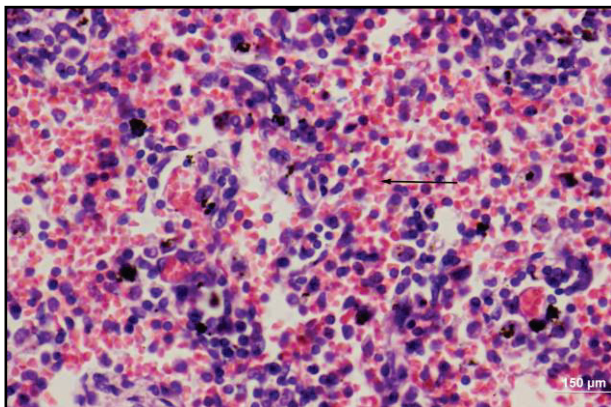


Fig. 6. Fragment of microscopic structure of the cortex of intestinal lymph node of an experimentally infected puppy: erythrocytes between lymphocytes (indicated by arrow); hematoxylin and eosin

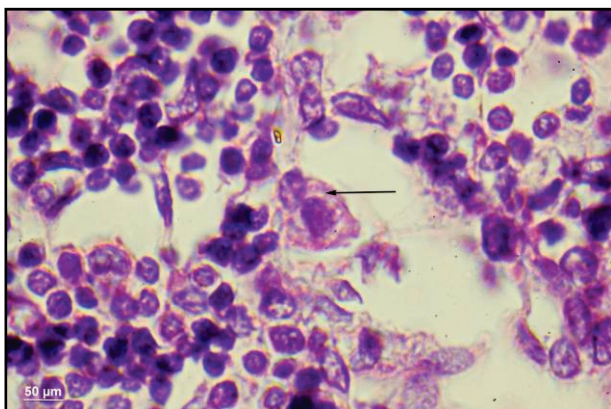


Fig. 7. Fragment of microscopic structure of the cortex of portal lymph node of an experimentally infected puppy: close contact between macrophage and lymphocyte (indicated by arrow); hematoxylin and eosin

Breakdown of a large amount of erythrocytes leads to accumulation of granules and grains of hemosiderin in the intercellular space of the red pulp, which was also found in large amounts in the cytoplasm of numerous macrophages (siderophages, Fig. 10). In the red pulp, we observed proliferation of reticular cells. During the histochemical reactions, in the places of breakdown of erythrocytes in the organ's parenchyma, we saw accumulation of acidic proteins (Fig. 11). Lymphoid nodules underwent noticeable microscopic changes as well. Part of them were quite expressive, but arranged eccentrically in relation to their central arteries (Fig. 12).

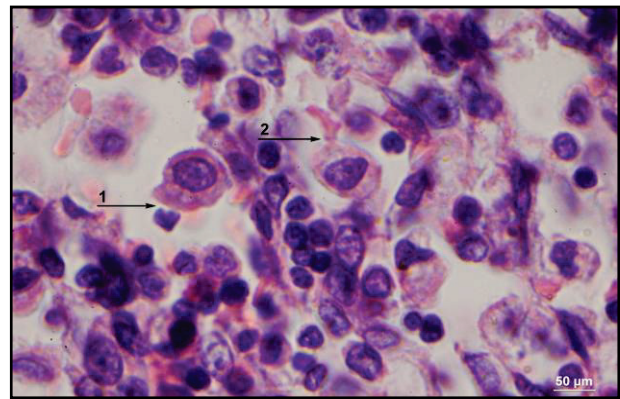


Fig. 8. Fragment of microscopic structure of the medulla of intestinal lymph node of an experimentally infected puppy: 1 – contact between macrophage and lymphocyte; 2 – contact between macrophage and irregular-shaped erythrocyte; hematoxylin and eosin

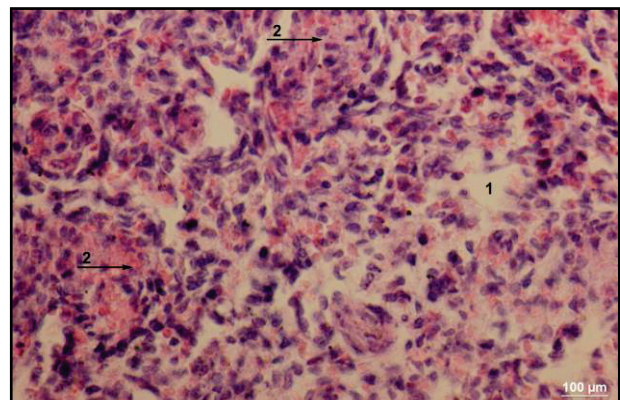


Fig. 9. Fragment of microscopic structure of the spleen of an experimentally infected puppy: 1 – edema of the red pulp; 2 – massive ruination of erythrocytes; hematoxylin and eosin

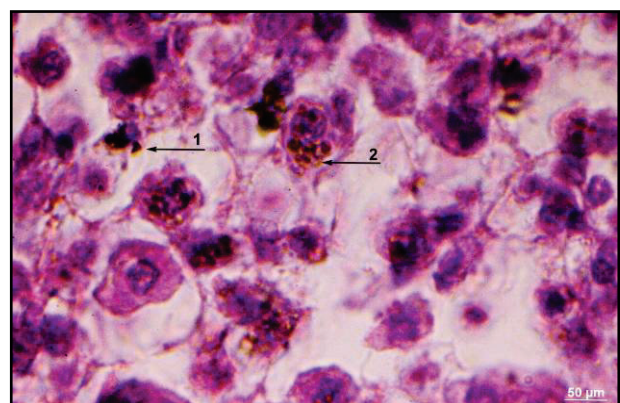


Fig. 10. Fragment of microscopic structure of the red pulp of the spleen of an experimentally infected puppy: 1 – hemosiderin in the intercellular space; 2 – hemosiderin in cytoplasm of hemosiderophage; hematoxylin and eosin

Rest lymphoid nodules were large, usually having indistinct edges (Fig. 13). In most lymphoid nodules, we found quite a large number of macrophages among lymphocytes. Single macrophages were necrotized (Fig. 14). Some lymphocytes that localized within lymphoid nodules, as well as among other cells of the red pulp, contained eosinophilic inclusions in their nuclei (Fig. 15).

Discussion

One of the most important directions of the development of modern veterinary practice is improvement and introduction of modern methods

of diagnostics and predicting the course of disease based on determining the level of deterioration of functional activity and possibility of correction of protective functions of the organism. Parvoviral enteritis of canines has been known during the last forty years. Despite such a long time, unfortunately, no drugs for its treatment and prevention exist as of now, and taking into account resistance of the virus in the environment and its ability to mutate, correlation of diagnostic methods is always necessary, and therefore studies of parvoviral infection in dogs are relevant.

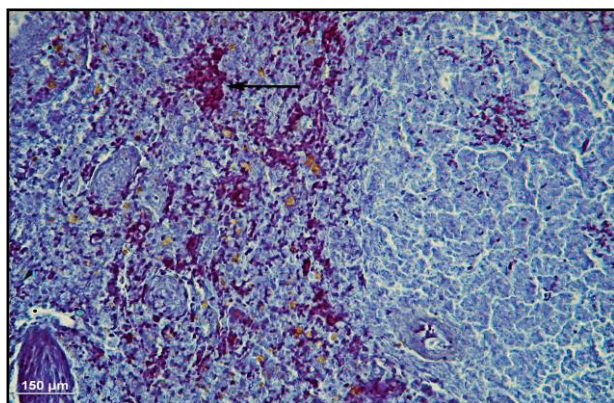


Fig. 11. Fragment of microscopic structure of the spleen of an experimentally infected puppy: accumulation of acidic proteins in places of breakdown of erythrocytes (indicated by arrow); Michel-Calvo technique

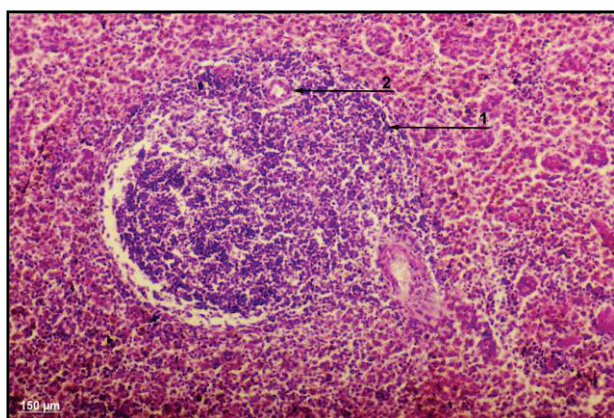


Fig. 12. Fragment of microscopic structure of the spleen of an experimentally infected puppy: 1 – lymphoid nodule; 2 – central artery; hematoxylin and eosin

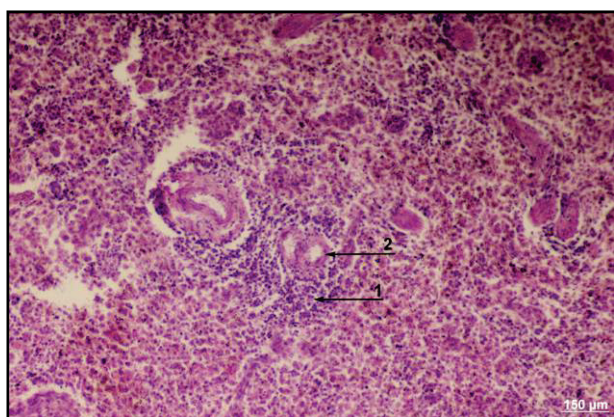


Fig. 13. Fragment of microscopic structure of the spleen of an experimentally infected puppy: 1 – lymphoid nodule; 2 – central artery; hematoxylin and eosin

During pathomorphological study of the corpses of all animals, we observed expressed macroscopic changes in the immune-competent organs of hemo- and immunopoiesis, which manifested in destructive

changes, inhibition of the immunogenesis function during the infectious disease, which correlates with the data on pathogenesis (Schoeman et al., 2013; Ford et al., 2017) and the analysis of pathomorphological results obtained by scientists (Goddard & Leisewitz, 2010; Fagbohun et al., 2020), and thus those changes may be considered pathomorphologic.

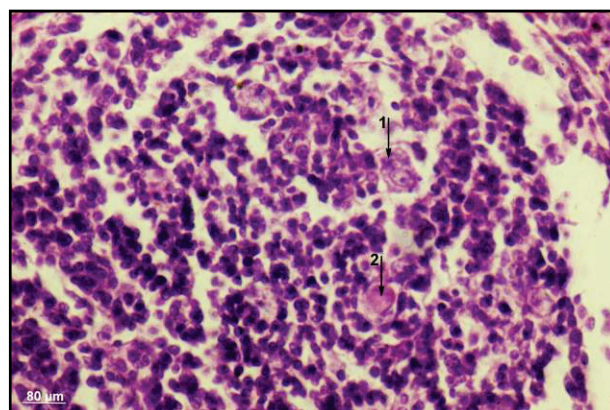


Fig. 14. Fragment of microscopic structure of splenic lymphoid nodule of an experimentally infected puppy: 1 – macrophage with vacuole in cytoplasm; 2 – necrotized macrophage; hematoxylin and eosin

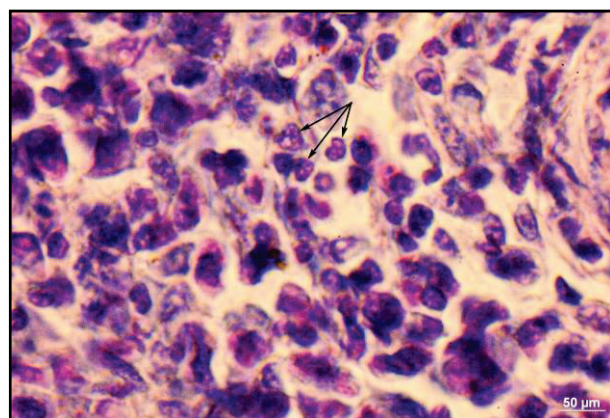


Fig. 15. Fragment of splenic lymphoid nodule of an experimentally infected puppy: lymphocytes with intranuclear eosinophilic inclusions (indicated by arrow); hematoxylin and eosin

Notable microscopic changes were found in all lymphoid organs and tissues. As a result of our studies, which are to a certain degree coherent with the studies by Mazzaferro (2020), we determined inhibition of realization of cellular link of immunity as a result of autophagy of the thymus which blocks the synthesis of cytotoxic cells, which play an important role in the protection of the organism from pathogenic action of viruses. Furthermore, T-cells synthesize lymphocines (cytokines): interleukins, interferon, which regulate the function of macrophages and other lymphocytes. In our opinion, disorganization of thymic corpuscles resulted in small sites of necrosis of lymphocytes in the thymus. As reported in studies of Pereira et al. (2019), an important role in combating infectious antigen is performed by antigen-presenting T-lymphocytes, which connect the inherited and adaptive immune responses to an alien agent. This necessary immune function is impaired during pathological changes in the thymus.

In all somatic and visceral lymph nodes, most lymphoid nodules had no light centers. Instead, there were quite large sites of necrosis of lymphocytes in the central areas of most of them. Sizes of lymphoid nodules of the stomach and single and concentrated lymphoid nodules of the small intestine and the large intestine were notably decreased, they were found to have sites of necrosis of lymphocytes. Some lymphocytes in the thymus, lymph nodes, lymphoid nodules of the spleen, in lymphoid nodules of the stomach, single and concentrated lymphoid nodules of the small and large intestines contained intranuclear eosinophilous inclusions, which is coherent with the results of other scientists (Geetha, 2015; Mylonakis et al., 2016; D'Oliveira et al., 2018).

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