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Investigating hydropericardium syndrome with different histopathological techniques in broiler chickens

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Abstract

Hydropericardium syndrome (HPS) has caused significant financial losses to the Iranian poultry industry in the past few years. Thirty-two broiler chickens with gross lesions of HPS were inspected histologically and immunohistochemically. Sampling was performed in Sabzevar, Iran. The dead and sick birds from random farms were subjected to necropsy examinations. Only four broiler chickens had no hydropericardium and the other gross findings were similar for birds. Basophilic and eosinophilic intranuclear inclusions, hemorrhages and necrosis in different organs were the primary characteristic histologic lesions. Lymphoid depletion, goblet cell hyperplasia and necrotizing enteritis were some of the findings reported in previous research. Low macrophage infiltration rate and brain lesions were other discoveries in Hematoxylin and Eosin (H&E) examination. Feulgen reaction and Cluster of Differentiation 68 (CD68) immunohistochemical staining were used for a comprehensive investigation and these techniques revealed improved histopathologic details. Feulgen staining confirmed brain lesions and some other changes in different organs. Eventually, the CD68 method revealed low macrophage presence in most organs. This study suggested that HPS might cause brain damage and the susceptibility of the Arian breed to the adenovirus needs further investigation.

Introduction

Hydropericardium syndrome (HPS) is a remarkably infectious disease initially reported in Pakistan, however, is spreading in other countries.[1] Fowl adenoviruses (FAdV) belong to the Aviadenovirus genus and are the causative agents of the disease.[2] The HPS was thought to be caused by nutritional deficiency or toxicity. Nonetheless, all the efforts to reproduce the disease experimentally using different toxic agents were unsuccessful.[1]

Fowl adenovirus 4 (FAdV-4) is the causative agent of HPS. This serotype is non-enveloped and has an icosahedral shape, measuring 70.00 - 90.00 nm in size and containing a linear double stranded DNA. The FAdV-4 serotype affects poultry and causes substantial financial losses to the poultry industry.[3]

The HPS was also called Angara disease because it was reported primarily in Angara Goth, Karachi, Pakistan, in August 1987.[4] It was reported in Iraq in 1991.[5] Three years later, Jammu and Kashmir of India reported the disease.[1] Litchi heart disease is the other name for this syndrome.[6] Considering that the disease is associated with liver damage and has a similar causative agent as inclusion body hepatitis another name for the disease is hepatitis-hydropericardium syndrome (HHS).[6]

The HHS is mainly detected in the broilers of the age group of 3 - 5 weeks. It is transmitted horizontally among chickens and it has mechanical route including vaccinators and litter contaminated with infected feces. The sudden onset of high mortality up to 75.00% is observed while finding no other clinical signs. Nevertheless, some birds are occasionally depressed, huddle in corners, and have ruffled feathers before death.[5]

The HPS virus spreads rapidly from farm to farm or flock to flock. Hepatic, endothelial, and lymphatic cells are the main targets of the virus. Marked destruction of erythrocytes, particularly in the spleen and lungs, has been observed in experimental and natural cases.[1]

Hydropericardium and hepatic necrosis have a close correlation. Marked hepatic circulatory failure may be due to acute hepatic necrosis. Heart circulatory failure and hydropericardium can occur because of this hepatic

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circulatory failure. Acute hepatic necrosis leads to acute heart failure and finally death. Ascites syndrome in broilers supports this hypothesis.[7]

The disease is characterized by a swollen pericardial sac filled with straw-colored fluid.[4] The accumulation of transparent jelly-like fluid in the pericardium is often associated with swollen, friable and discolored livers. Kidneys are congested at gross examination.[6] Petechial and ecchymotic hemorrhages in the heart musculature are found in the heart and other organs, and the lungs are associated with edema and congestion.[5]

Different histologic lesions exist on various organs; however, the most characteristic lesion is on the liver. There are basophilic and eosinophilic intranuclear inclusion bodies in hepatocytes. Multifocal necrosis of hepatic cells, mononuclear cell infiltration and hemorrhages are detected in the liver.[8] Numerous macrophages with yellow pigments are observed in the sinusoids of the livers of some chickens.[7]

The inclusion bodies are distributed in various organs such as the liver, gizzard, pancreas, duodenum, proventriculus, cecum and kidney. Nephritis, glomerulonephritis, nephrosis and extensive hemorrhages are found in some chickens.[1]

The pancreas shows necrotic foci and multifocal necrosis of the pancreatic acinar cells. Intranuclear inclusions in some of the degenerative cells around necrotic foci and the infiltration of macrophages and lymphocytes in the necrotic foci are the other observations in the pancreas. Lymphocyte depletion, a noticeable increase of macrophages, deposition of yellow pigment in splenic sinuses and fragmentation of erythrocytes are observed in the spleen. The epicardium demonstrates mild serous exudation; however, the myocardium has no lesions. Several macrophages with yellow pigment are found between the air capillaries in the lungs. These air capillaries have narrow lumens.[7]

The HPS is difficult to diagnose since birds do not show definite clinical signs. Therefore, the diagnosis is based on gross lesions as well as histopathologic and ultrastructural evaluations.[6] Immunohistochemistry and special staining such as the Feulgen technique have been used in some studies. Feulgen staining is especially for DNA and can be employed to confirm the presence of viruses in the liver.[4]

The isolation of the virus using embryonated eggs or cell culture and aneutralization test can be helpful. Immunodiagnosis by serological tests like counterimmunoelectrophoresis, dot immunoblot assay, modifications of enzyme-linked immunosorbent assay and fluorescent antibody technique is also reliable. The polymerase chain reaction using specific primers is another diagnostic method.[6]

The virus is resistant to disinfectants and vaccination is the only choice to protect birds against HPS. Montanide adjuvanted vaccines, autogenous formalin-inactivated liver homogenate vaccines, oil-emulsified vaccines, recent vaccines propagated in cell culture systems and embryonated chicken eggs have been developed and used in different countries.[9]

Considering that adenovirus has caused significant financial losses to the Iranian poultry industry in recent years, the pathological characteristics of this virus should be examined more closely. The study extensively investigated microscopic changes of Angara disease in broiler chickens. Furthermore, some special staining was used to obtain more details about the disease.

Materials and Methods

Broiler chickens showing a colored fluid in the pericardial cavity were collected for histopathologic evaluations. Sampling was performed in Sabzevar, a city located in eastern Iran. Dead and recumbent birds were subjected to necropsy. After a postmortem examination, tissues including liver, heart, kidney, pancreas, duodenum, proventriculus, lung, gizzard, brain and spleen were collected and fixed in 10.00% buffered formalin.

The Hematoxylin and Eosin (H&E) technique was performed as routine for the histopathological study. The tissue samples were embedded in paraffin, sectioned at 4.00 µm thickness and stained with H&E. Then, two methods were employed for detailed evaluation and definite diagnosis, namely, an immunohistochemical marker for macrophage detection Cluster of Differentiation 68 (CD68) and special staining for demonstrating DNA in tissue sections (Feulgen stain).

In routine Feulgen stain, the slides are immersed in 5.00 mol L^{-1} HCl for 15 min. Then, they were rinsed with distilled water for 3 min, washed for 10 min and stained with Schiff's reagent (Amber Glass Winchester, Winchester, UK) for 90 min. For the final step, the slides were rewashed for 10 min and stained with 1.00% of light green for 15 min.

The CD68 staining was utilized for macrophage infiltration examination. Staining performed using a commercial Kit (CD68 Monoclonal Antibody clone KP-1; Master diagnostic, Sevilla, Spain) according to the manufacturer's instructions. A 1.00 µm tissue section was placed on the poly-l-lysine coated slides. These slides were deparaffinized in three baths of xylene for 5 min each. Next, they were rehydrated with alcohol and distilled water. Antigen retrieval was performed with the Tris-ethylenediaminetetraacetic acid solution at 98.00 ˚C for 20 min. After cooling down in the buffer, a tris-buffered saline (TBS) solution was used for 35 min. Then, an H_2O_2 solution was applied to inhibit the background reaction. The slides were incubated with a primary antibody for 30 min at room temperature. Subsequently, a post-primary block was employed for 20 min and the slides were incubated with Novolink polymer for another 20 min. The TBS solution was used for rinsing between the steps and the slides were

incubated with diaminobenzidine. Finally, the nuclei were counterstained with Hematoxylin for 3 min. After the rehydration, the slides were mounted for microscopic (B-190; Optika, Ponteranica, Italy) examination.

Results

Clear straw-colored fluid in the pericardial cavity was the typical characteristic gross lesion of our study. Hydropericardium was observed in most of the birds. Only four of 32 broiler chickens did not display hydropericardium in the heart. In all cases, the livers were enlarged (Fig. 1) and hemorrhages were detected in some of the birds. Different parts of the intestine, lungs and kidneys of some birds showed hyperemia and no other findings were recorded on gross examination.

Fig. 1. A) Clear straw-colored fluid is observed in the pericardial cavity, and **B)** enlarged liver lobes are seen.

Based on microscopic examinations, basophilic intranuclear inclusion bodies were seen in different portions of liver (Fig. 2A). Eosinophilic intranuclear inclusion bodies were also present in a few numbers. Liver necrosis had a diffuse pattern and was primarily found around the vessels. Many hepatocytes with inclusion bodies were enlarged to several times the size of normal hepatocytes. Cardiomyocyte necrosis and loss of striation, infiltration of mononuclear cells, shortening of muscle fibers and congestion were observed in heart. The hyperplasia of adipocytes and vacuolar degeneration of cardiomyocytes were detected and epicardium illustrated fibrinous and serous exudation (Fig. 2B). The brain had widespread perivascular and perineuronal edema (Fig. 2C). Ischemic cell change was another finding (Fig. 2D) and its severity varied among birds. Epithelial tubular cells in kidney were associated with degeneration and necrosis. Intranuclear basophilic inclusion bodies were also present in these cells (Fig. 2E). Glomerulonephritis was observed in kidney and extensive hemorrhage was found in different portions. There was hyperemia and extensive hemorrhage in lung. Fibrinous exudate and severe inflammation were also detected in lung; however, macrophage infiltration was hardly observed at H&E staining (Fig. 2F). Intranuclear inclusion bodies were observed in pancreas, with no extensive or focal necrosis (Fig. 2G). Gizzard, similarly to pancreas displayed inclusion bodies, also. Intranuclear inclusion bodies in gizzard were mostly eosinophilic and basophilic types were also present (Fig. 2H).

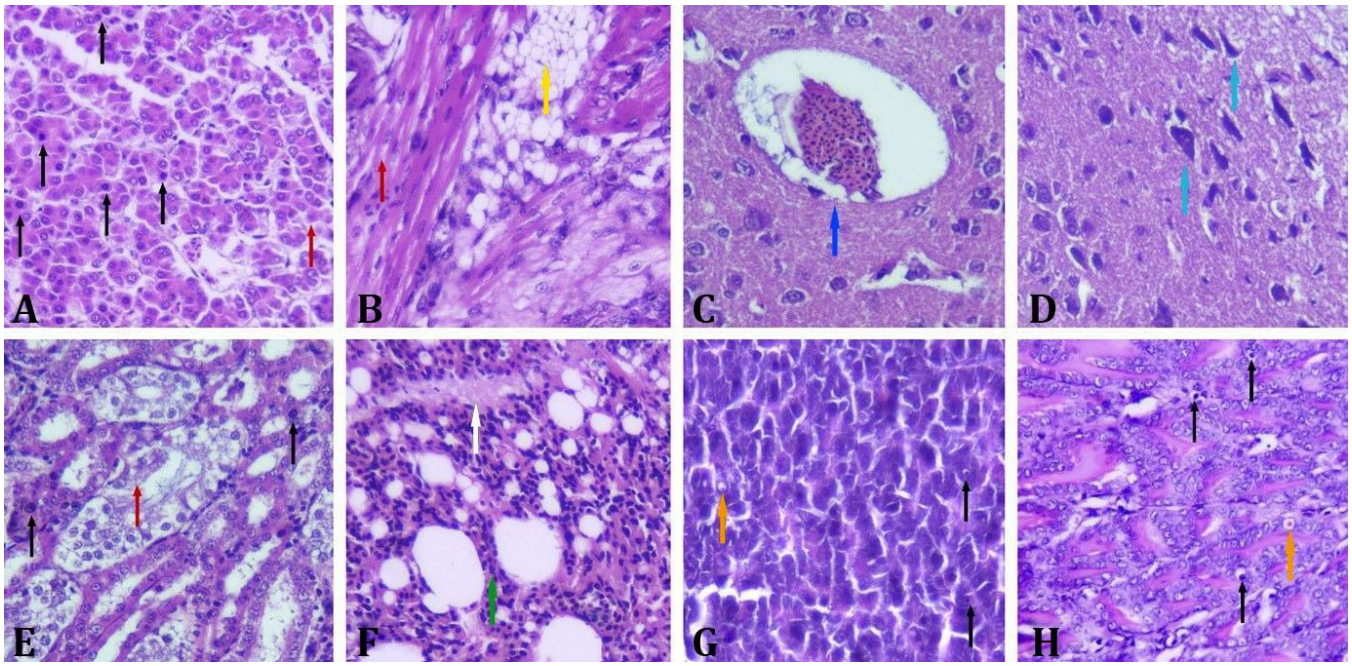

Fig. 2. The histopathological alterations of different tissues stained. **A)** Liver, hepatocyte necrosis (red arrow), **B)** Heart, adipocyte hyperplasia (yellow arrow), and necrosis (red arrow), **C)** Brain, perivascular edema (blue arrow), **D)** Brain, ischemic cell change, and perineuronal edema (turquoise arrows), **E)** Kidney, epithelial cell necrosis (red arrow), **F)** Lung, inflammatory cells (green arrow), and fibrinous exudation (white arrow), **G)** Pancreas, eosinophilic intranuclear inclusion body (orange arrow), and **H)** Gizzard, eosinophilic intranuclear inclusion body (orange arrow). Black arrows show intranuclear basophilic inclusion bodies (H&E staining; 400×).

The necrosis of proventriculus glands and mucosal necrosis were detected and inclusion bodies were also recorded in this tissue.

Lymphatic drainage was documented in the spleen of birds. The spleen showed eosinophilic intranuclear inclusion bodies. These bodies were recorded in different parts of the intestine including the duodenum. Goblet cells had hyperplasia and inclusion bodies were detected in duodenal epithelium from the glands to villous tips. There was necrotizing enteritis in the duodenum.

Feulgen staining confirmed the H&E staining findings and verified more details. Intranuclear inclusion bodies were also observed in Feulgen staining. More inclusion bodies were recognized in this technique. The epithelium of renal tubules, different portions of the kidney and the intestine were also associated with inclusion bodies. Some cells such as cardiomyocytes had irregular nucleoli (Fig. 3).

The CD68 immunohistochemical staining was performed to examine macrophage infiltration. According to this staining, macrophage infiltration in different tissues was low. However, the presence of tissue macrophages and their debris in the lungs, kidneys, liver, heart, spleen and brain was recorded in various numbers (Fig. 4).

Discussion

In recent years, Angara has caused economic losses to the breeding farms in Iran. Various provinces have experienced significant financial losses due to HPS and Khorasan Razavi province in the east of the country was one of them. The clinical symptoms of birds affected by this disease were not much different from previous researchers report. The mortality rate of birds was extremely high and sudden death was reported accordingly. Based on field observations, the mortality of some Iranian herds reached to 80.00%.

The most common gross lesion of Angara disease is hydropericardium that was consistent with our findings. Amber-colored and jelly-like fluid in the pericardial sac was accumulated in all birds except for four cases that showed no specific hydropericardium in the heart.[8,10,11] The liver was enlarged and friable, similar to a previous study,[4] and petechial hemorrhages and necrotic areas were observed in the liver.

Microscopically, the lesions in this research were similar to most prior experiments. Necrosis, infiltration of macrophages and mononuclear cells, loss of striation and congestion were observed in the heart that were consistent with previous research.[12]

According to a report by Niu *et al*.,[12] the infiltration of mononuclear cells and the extravasation of erythrocytes between the heart muscle fibers were also observed in our study. Similar to Nakamura inquiry,[4,7] epicardium showed fibrinous and serous exudation and myocardium indicated an eosinophilic change of myocardial fibers and hemorrhages. In the present study, vacuolar degeneration was also present in the heart compatible with previous research.[7] Despite the severe weight loss, previous studies did not report adipocyte hyperplasia.

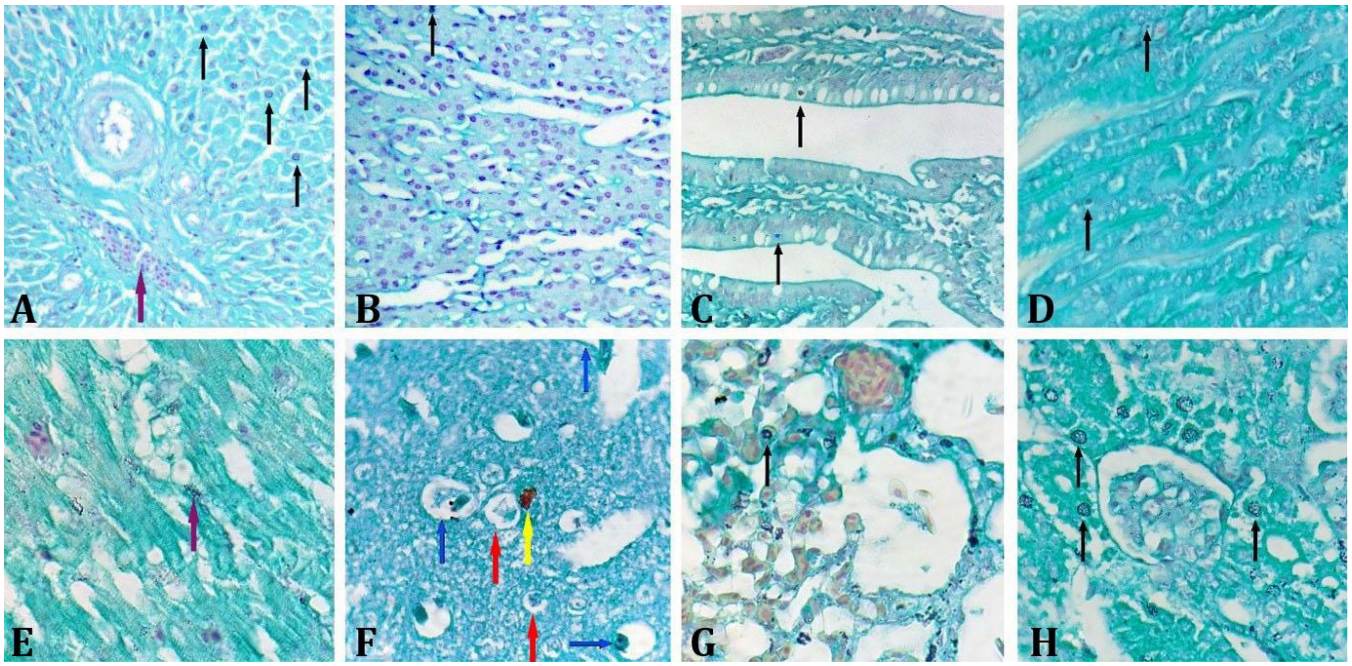

Fig. 3. The histopathological (Feulgen staining) alterations of different tissues. Black arrows illustrate intranuclear basophilic inclusion bodies. **A)** Liver (400×), hyperemia (pink arrow), **B)** Pancreas (400×), **C)** Duodenum (400×), **D)** Gizzard (400×), **E)** Heart (1,000×), irregular nucleoli of cardiomyocytes (purple arrow), **F)** Brain (1,000×), perivascular edema (blue arrows), perineuronal edema (red arrows), and foreign body giant cells (yellow arrow), **G)** Lung (1,000×), and **H)** Kidney (1,000×).

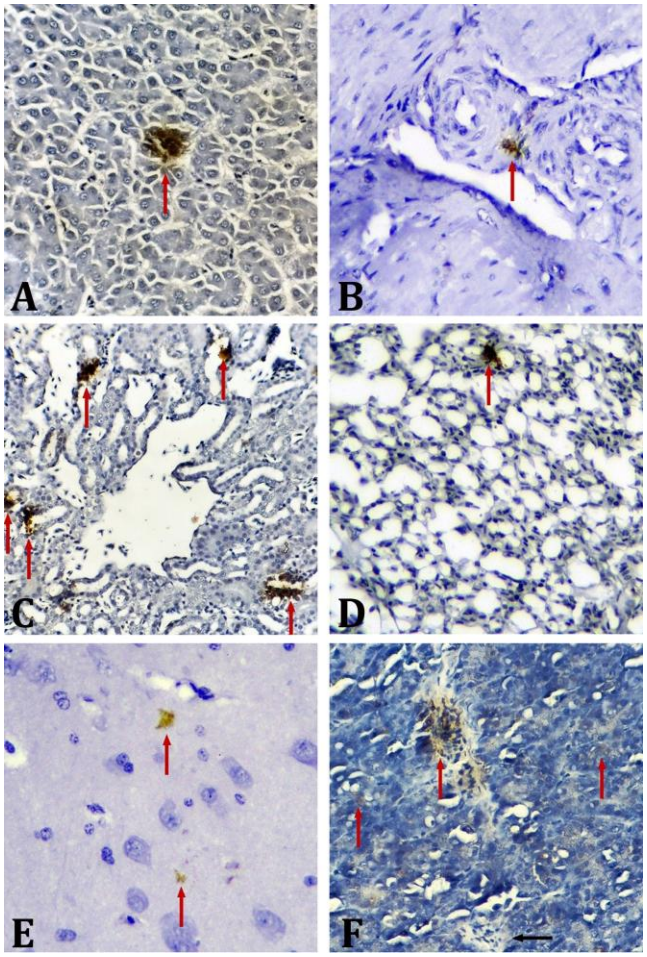

Fig. 4. The histopathological alterations of different tissues at Cluster of Differentiation 68 examination: **A)** Liver, **B)** Heart, **C)** Kidney, **D)** Lung, **E)** Brain, and **F)** Spleen, lymphoid depletion (black arrow). Red arrows depict macrophages (400×).

Generally, the inclusion bodies were distributed in the liver, pancreas, gizzard, proventriculus, duodenum and kidney as reported by some previous surveys.[10,13]

Diffuse hepatocellular necrosis and basophilic or eosinophilic intranuclear inclusion bodies were observed in different portions of the liver. Contrary to studies by Abe *et al*.[4] and Nakamura *et al*.[7,8], basophilic inclusions were more common than eosinophilic ones. Nakamura *et al*.[7] revealed that many hepatocytes with inclusion bodies were enlarged to several times the size of unaffected hepatocytes, which were in agreement with our study results. Unlike the findings of Nakamura *et al*.[7], there was no macrophage with yellow pigments in the sinusoids of the livers.

However, in our study, diffuse infiltration of mononuclear cells was another finding in the liver which was consistency with the findings of Niu *et al*.[12]

In our study, the kidney displayed the degeneration and necrosis of tubular epithelial cells, similar to the investigation by Niu *et al*.,[12] however, there was no edema around the tubular epithelium. In our examination, glomerulonephritis and extensive hemorrhage were the other lesions documented in the kidney. In our study, intranuclear basophilic inclusion bodies in the tubular epithelium were more comparable to the study by Nakamura *et al*.[7]

Lungs showed hyperemia, extensive hemorrhage, fibrinous exudate and severe inflammation. Contrary to most other studies,[4,7,8] macrophage infiltration was not recorded in H&E staining. Abe *et al*.[4] reported that macrophages had engulfed yellow pigments in the air capillary and capillary blood areas, however, no pigment was found in our findings. The results related to congestion and edema in the lung were consistent with the result of Niu *et al*.[12]

Despite previous studies,[4,7] a few macrophages were observed in the splenic sinuses. Other researchers[4,7] reported erythrophagocytosis (yellow pigments) or fragmented erythrocytes in splenic macrophages which contradicted the results of our study since we did not detect them in the spleen. Conversely, Nakamura *et al*.[7] revealed that lymphatic drainage and eosinophilic intranuclear inclusion bodies were documented in the spleen.

Dissimilar to the studies by Nakamura *et al*.[7,8], there was no extensive or focal necrosis in pancreas, and basophilic intranuclear inclusion bodies were the only finding.

Intranuclear inclusion bodies in gizzard were eosinophilic, however, consistent with the findings of the research by Nakamura *et al*.,[7,8] basophilic types were also present. The necrosis of proventriculus glands was detected and desquamated epithelial cells with intranuclear inclusions were recorded as well, which was in agreement with the findings of Nakamura *et al*.[7]

Similar to the other portions of the intestine, the duodenum also displayed basophilic inclusion bodies. They were detected in duodenal epithelium from the glands to villous tips, which was in conformity with the results of Nakamura *et al*.[7,8]

In agreement with Abe *et al*.,[4] in our study, the Feulgen reaction was used to confirm the inclusion bodies and the intranuclear inclusions showed positive staining by Feulgen staining. The staining was positive for the liver and kidney but also demonstrated practical details in the other organs. Brain lesions were observed in this study, a new finding that was not reported in other studies.

The lesions in the present study confirmed the possible sensitivity of the Arian breed to the virus. However, the correlation between these brain lesions and the Arian breed requires a more detailed investigation. For this reason, other discrepancies in our findings could also be attributed to breed differences.

Considering that the spread of adenovirus in the body is rapid, there is not enough time for macrophage infiltration. However, neutrophils acting in the acute phase of the reaction are visible.

Macrophages enter the sites of inflammation shortly after neutrophils and there is not much time difference between them.[14] However, adenovirus does not seem to allow the proper activity of the immune system in the Arian breed. Broad and rapid mortality in the studied farms confirms this assumption. The low number of macrophages could also be attributed to the sensitivity of the Arian breed to Angara; therefore, more research is recommended in this respect.

Conflict of interest

The author states that there are no conflicts of interest in relation to the publication of this article. The research and findings presented have been conducted with a focus on integrity and transparency, ensuring that personal or financial affiliations do not affect the outcomes or interpretations of the work.

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