

Structural-functional changes in the proventriculus of poultry infected with transmissive viral proventriculitis

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Transmissive viral proventriculitis (TVP) is a new avian disease, the etiology and pathogenesis and diagnostics of which remain unidentified, being therefore a subject of an active ongoing discussion among researchers and veterinary doctors. Cases of the disease have been reported and registered in many countries of the world. On poultry farms of Ukraine, this disease started to be diagnosed in 2019, according to the characteristic pathognomic changes. The article presents the results of pathoanatomical, histological and electronic-microscopical studies of the glandular part of the stomach of 62 ill broiler chickens aged 24–28 days and 7 clinical healthy broiler chickens of the same age. The samples of the proventriculus from the infected and clinically healthy chickens were fixed in 10% aqueous solution of neutral formalin and in 1.5% solution of glutaraldehyde in 0.2 molar cacodylate buffer (pH 7.2). Pathoanatomic necropsy of the infected broiler chickens revealed overall anemia, decrease in the volume of the spleen, thymus and fecal sac. In the glandular part of the stomach, the wall was slightly thickened, non-homogenously stained on the section, the mucous membrane being swollen, covered by dense whitish mucous. According to the histological study, the proventriculus of the broiler chickens had lymphocytic necrotizing proventriculitis. In the tubular-alveolar structures of the mucous membrane, there prevailed alteration of the secretory epithelium, with its transformation into ductal epithelium, combined with lymphocytic infiltration of the interstitium. Intensive necrosis of most glandular (oxynticopeptic) cells was accompanied by their exfoliation from the basal membrane of the secretory parts, with desquamated cells accumulating in enlarged lumens of alveoli and excretory ducts. Further, necrotized oxynticopeptic cells in the tubular-alveolar glands were actively replaced by cylindrical ductal cells, and also their hyperplasia and hypertrophy occurred. Diffusive and multifocal interalveolar round-cell infiltrates were mostly presented by lymphocytes, among which there occurred plasmocytes, macrophages, and heterophils. Ultrastructural studies of secretory cells revealed presence of pathogen virions in nucleus and cytoplasm. Based on the morphological changes in the birds' proventriculus, we diagnosed transmissive viral proventriculitis, which had not been recorded in Ukraine before.

Keywords: avian TVP; proventriculus; pathoanatomical changes; histopathology; ultrastructural changes.

Introduction

The emergence and spread of infectious diseases on poultry farms are extremely dangerous, and therefore timely diagnostics and prophylaxis are crucial (Li et al., 2018; Kotsyumbas et al., 2019; Mazurkiewicz & Wieliczko, 2019). Since 2019, on some poultry farms of Ukraine, there has been an increasing incidence of a disease in the broiler chickens (especially older than 3-years old) that manifested in stunted growth and development, decrease in mean-daily increments and body weight, and deterioration of the carcasses' appearance. Pathoanatomical studies found no changes characteristic of a certain disease, except signs of inflammation of the glandular stomach, which raised suspicions of transmissive viral proventriculitis (TVP), which had not been recorded in Ukraine before.

In the sphere of industrial poultry farming, the raising of broiler chickens having TVP becomes cost-worthy and economically unprofitable. The disease can affect up to 50% of the flock population. According to the data of some researchers, the incubation period is 5–7 days, while according to the others it can take up 10–14 days. Mortality from this disease is low – up to 5%, but the number of cull chickens increases (Kouwenhoven et al., 1978; Goodwin et al., 1996; Guy et al., 2007).

The first reports about TVP of broiler chickens were made by a group of Dutch researchers in 1978, who described the disease and assumed its viral etiology. From the tissues of a sick bird, a non-cellular filtrate was obtained and a pathogen was isolated, which likely belonged to the adenoviruses (Kouwenhoven et al., 1978).

The disease has been diagnosed ever since among poultry on farms of the USA (Guy et al., 2005), France (Marguerie et al., 2011), Australia, China (Li et al., 2018), South Korea (Kim et al., 2015), Spain (Grau-Roma et al., 2010), Great Britain, and Poland (Grau-Roma et al., 2017, 2020; Smialek et al., 2017).

As of now, the viral etiology of the disease is still unidentified. Transmission electronic microscopy revealed presence of hexagonal viral particles in nuclei and cytoplasm of cells of the proventriculus, though the method of *in situ* DNA hybridization found no nucleic acids of adenovirus and polyomavirus (Goodwin et al., 1996). Since 2011, the majority of researchers have stated that the TVP-causing virus belongs to the *Birnaviridae* family. A group of American scientists conducted a number of experiments, discovering virions of the pathogen in the cells of the glandular part of the stomach of spontaneously and experimentally infected birds. An assumption was made that this is a new representative of the *Birnavi-*

ridae family – chicken proventricular necrosis virus (CPNV), which is different from infectious bursal disease virus (IBDV) of the *Avibirnavirus* genus (Guy et al., 2011). Soon afterwards, other researchers confirmed this hypothesis that the virus of the *Birnaviridae* family, responsible for emergence of proventricular necrosis, is also the pathogen of TVP and those diseases are closely related to each other (Marguerie et al., 2011; Noiva et al., 2015; Grau-Roma et al., 2017). At the same time, researchers discuss etipathogenesis of TVP, assuming a possible participation of gyrovirus 3 (GyV3) of the *Anelloviridae* family (Li et al., 2018), novel cyclovirus chCyCV-SDAU-1 of the *Circoviridae* family (Yan et al., 2020), and novel chicken picornavirus, which is close to turkey viral hepatitis and chicken megrivirus of the *Picornaviridae* family (Kim et al., 2015), and also accompanying fungal or bacterial infections.

A characteristic of TVP is the development of necrotizing inflammation of the poultry proventriculus, observed at the histological level. During necropsy of chickens that died from TVP, this process is hard to identify by macroscopical signs even for an experienced veterinary specialist. Therefore, according to most researchers, the most reliable method of TVP diagnostics is histological study of the proventriculus (Guy et al., 2011; Grau-Roma et al., 2020).

Taking into account that there have been no studies focused on pathomorphological characteristics and diagnostics of avian TVP in Ukraine, our objective was to determine peculiarities of changes in the morpho-functional condition of the proventriculus of the broiler chickens, grown on farms of Western Ukraine, in cases of suspicion of transmissible viral proventriculitis.

Materials and methods

Studies on the chickens were performed according to the rules adopted by the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Strasbourg, 1986), the General Ethical Principles of Experiments on Animals, adopted by the 1st National Congress of Bioethics (Kyiv, 2001), the Law of Ukraine on Protection of Animals from Abuse, which outline certain conditions of maintenance, nutrition, and care for birds, and also performing euthanasia by a humane method.

In 2021, on three poultry farms in Lviv, Ivano-Frankivsk, and Volyn Regions of Ukraine, the poultry was observed to have a disease manifesting in stunted growth and development, as well as steep decrease in feed conversion. For a pathomorphological study, we selected 30 bird corpses, 32 clinically ill individuals with poor growth and development, of the Koob-500 cross, aged 24–28 days, and 7 clinically healthy birds of the same age and cross.

After the action of light ether narcosis, we euthanized the clinically healthy and infected chickens, performed their pathoanatomical necropsy and collected fragments of the proventriculus for a histological study. We collected samples and fixated them in 10% solution of neutral formalin, dehydrated them in ethanol of ascending concentration, consolidated and embedded in paraffin. The samples were prepared on an MS-2 microtome, stained with hematoxylin and eosin (Horalskyi et al., 2005). The preparations were photographed using the method of light microscopy employing a Leica DM-2500 microscope and a Leica DFC450C (Switzerland) photcamera and a Leica Application Suite Version 4.4 software pack. For an in-depth study of ultrastructure of cells of the mucous membrane of the proventriculus, the selected samples were fixated for 2 h in 1.5% solution of glutaraldehyde in 0.2 M cacodylate buffer (pH 7.2). The samples were rinsed in two portions of the buffer and completed fixation in 1.5% solution of osmium oxide (OsO_4). After rinsing and dehydration in ascending concentrations of ethyl alcohol, we contrasted them in uranyl-acetate and embedded in Epon-812 epoxy resin. Ultrathin sections were contrasted using uranyl acetate and lead citrate. The samples were examined and photographed using a PEM-100 transmission-electron microscope.

Results

During the pathoanatomical necropsy of the sick broiler chickens, we saw an overall anemia: whitish colour of the mucous membrane of the

oral cavity, esophagus, proventriculus, and the wall of the small intestine on the serous and mucous membranes. The liver was stained non-homogeneously: light-red or light-brown with light-violet spots and stripes, and weak consistency. The Glisson's capsule of the liver was smooth, shiny, and some petechial hemorrhages occurred (Fig. 1). In the proventriculus, ingesta was absent, the surface of the mucous membrane was mostly covered by dense white mucus, and in some chickens by mucus with pale-yellowish tone. The wall of glandular part of the stomach was insignificantly thickened, non-uniformly stained on the section: pale-violet or with light-red areas. When pressing the wall of the proventriculus with the finger, from the side of serous membrane, white dense clusters were extruded out of the lumens of some papillae of excretory ducts of the digestive glands of the mucous membranes (Fig. 2).

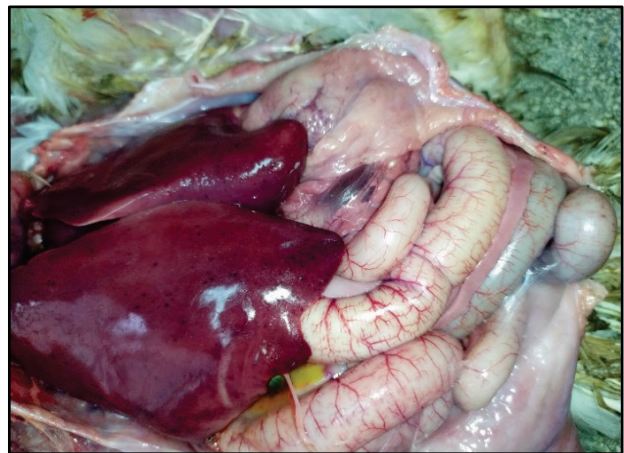


Fig. 1. Anemia of the serous membrane of the small intestine: petechial hemorrhages under the liver capsule; a 28-day-old broiler chicken



Fig. 2. Mucous membrane of the proventriculus: white clusters had been extruded from the lumens of papillae; a 28-day-old broiler chicken

Content was absent in the muscular section of the stomach, and the cuticle was greenish and easily removable. In the lumen of the small intestine, the content was absent, and the mucous membrane was white-grey, covered by dense white mucus. Compared with the clinically healthy birds of the same age group, the ill chickens were found to have decreased volumes of spleen, thymus, and Bursa of Fabricius. The spleen was rounded, of violet-grey tone, and weak consistency. The thymus was in the form of small greyish-reddish nodes.

Comparison of histological structure of the proventriculus of the sick chickens with such of the clinically healthy ones revealed pronounced structural-functional changes, which are currently considered pathognomonic and are diagnostically important. Histological study of the proventriculus of the clinically healthy chickens found that the superficial single-layered cylindrical epithelial of the mucous membrane and gastric pits was structured. The surface of the cells was covered by poorly eosinophilic

mucus, and the cells had distinct boundaries, eccentrically located basophilic nuclei, and poorly oxyphilous cytoplasm. In the mucous membrane, tubular-alveolar glands, appearing as multi-angular particles, were located in rows and separated one from another by thin layers of loose connective tissue (Fig. 3). The walls of the secretory sections were lined by a single-layered glandular epithelium (oxynticopeptic cells), mostly of cubic shape. Epithelial cells had distinct boundaries, eccentrically arranged nuclei, and intensively stained cytoplasm (Fig. 6). Epithelial cells of the tubular-alveolar glands are responsible for synthesis of pepsinogen and chlorides. Secondary and primary ducts were lined by columnar cells of the mucous membrane. Secretion of glandular cells enters the collective cavity of the area, and from there into the general excretory duct and is secreted to the surface of the mucous membrane, where it mixes with feed and travels the digestive tubule further for digestion.

In the sick chickens, the mucous membrane and tubular-alveolar glands underwent significant histostructural changes. The mucous membrane was thickened, the relief of its surface was disturbed, and the connective-tissue fibers were swollen, and loose. We saw disintegrated cylindrical epithelium of the mucous membrane and hyperplasia of the goblet cells. The gastric pits were filled with mucus and peeled-off epithelium (Fig. 4). In some places, we observed necrotic sites, and ruination of a large area of the superficial layer of the mucous membrane (Fig. 5).

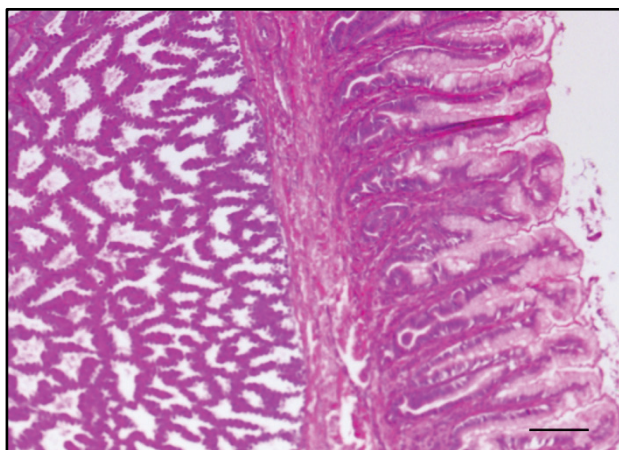


Fig. 3. Mucous membrane of the proventriculus of the healthy broiler chickens: hematoxylin and eosin; scale bar 200 μ m

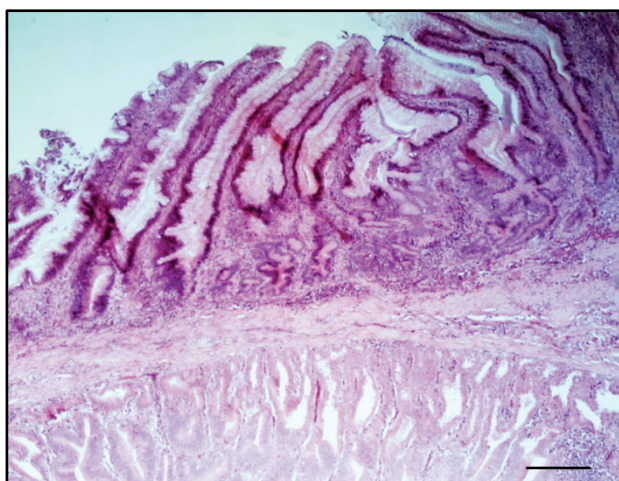


Fig. 4. Mucous membrane of the proventriculus of the TVP-suffering broiler chickens: gastric pits are filled with mucus; hematoxylin and eosin; scale bar 200 μ m

During the ultrastructural study of mucous membrane of the proventriculus, we found hyperplasia and hypertrophy of goblet cells, with close contacts between the cells remaining. They were much larger because of the cytoplasm was overfilled with secretory granules, varying by size and staining-colour intensity. Cell mitochondria were mostly oval, osmiophilic, with parallel cristae (Fig. 5). Ultrastructure of cell cytoplasm indicates

their increased functional activity, oriented at hypersecretion of mucus, which intensively coats the surface of mucous-membrane cells. A dense layer of mucus was often mixed with residuals of desquamated cells (Fig. 2).

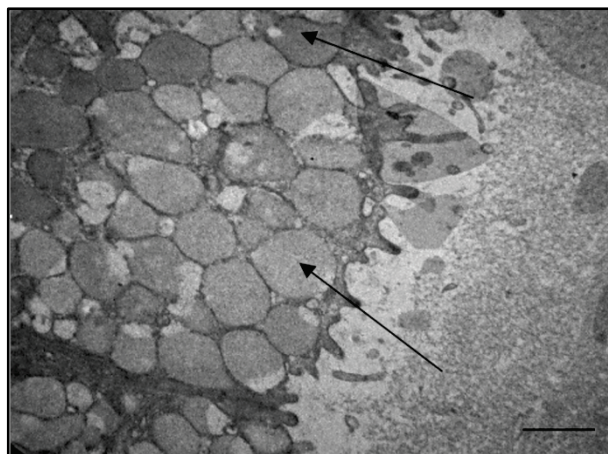


Fig. 5. Ultrastructure of goblet cell of the mucous membrane of the proventriculus of the TVP-suffering broiler chickens: cytoplasm is overfilled with secretory granules (indicated by arrows), varying in size and intensity; scale bar 500 nm

The palette of histological changes in various tubular-alveolar structures of the lamina propria of the mucous membrane of the proventriculus of the sick birds was not of the same type. Undamaged areas bordered with substantially altered glandular cells which underwent progressing alterative processes. Most cells of the secretory epithelium were swollen, with blurred contours, with amorphous, most often eosinophilic cytoplasm. Nuclei were in the states of rhexis and lysis (Fig. 7). Necrotized glandular cells were exfoliated from the basal membrane of the secretory walls and chaotically accumulated in alveolar lumens.

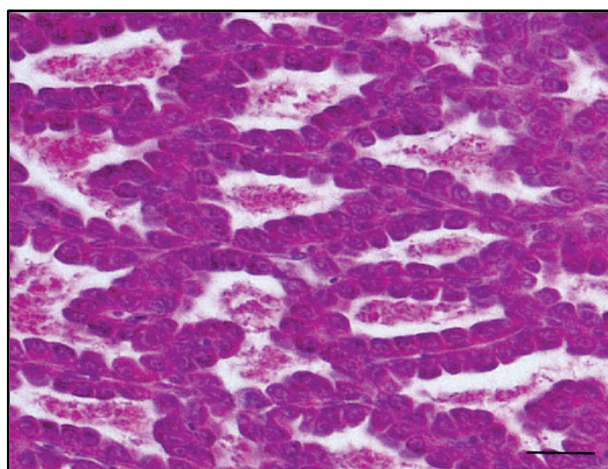


Fig. 6. Walls of secretory sections of the tubular gland of a healthy chicken: clearly structured glandular epithelium of the alveolar walls; hematoxylin and eosin; scale bar 50 μ m

Ultrastructural studies of the samples of proventriculus of the ill birds revealed loss of dense desmosome contacts of glandular cells, rupture of the connection with the basal membrane of alveoli. The range of changes in desquamated cells varied. In some cells, nuclei were mostly oval, located eccentrically, with decreased content of heterochromatin. The diffusely divided fine-dispersed karyoplasm chromatin had minimal pre-membrane condensation, which suggested steep decline in nuclear chromatin and nucleolus RNA. We should note that the nuclei of such cells contained accumulations of pathogen virions. Cytoplasm contained greatly enlarged cisterns of granular endoplasmic reticulum, which formed sac-like lumens (Fig. 8). Mitochondria were swollen, of rounded or oval-elongated shapes, mostly with fragmented cristae and cleared matrix. Along the configuration of mitochondria, there were dense membranes of endoplasmic reticulum with reduced quantity of ribosomes (Fig. 9).

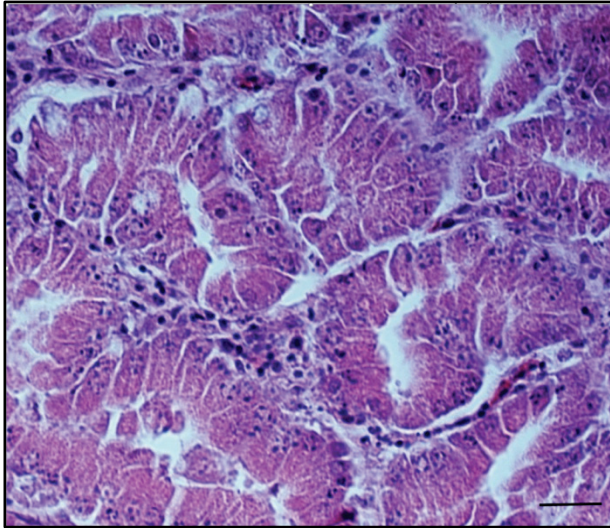


Fig. 7. Walls of the secretory sections of the tubular gland of a TVP-infected chicken: necrosis, disintegration, and separation of the glandular epithelium from the basal membrane, Karyorrhexis, lysis; hematoxylin and eosin; scale bar 50 μ m



Fig. 9. Glandular cell of the proventriculus of a TVP-infected bird: swollen mitochondria with fragmented cristae (a); scale bar 500 nm

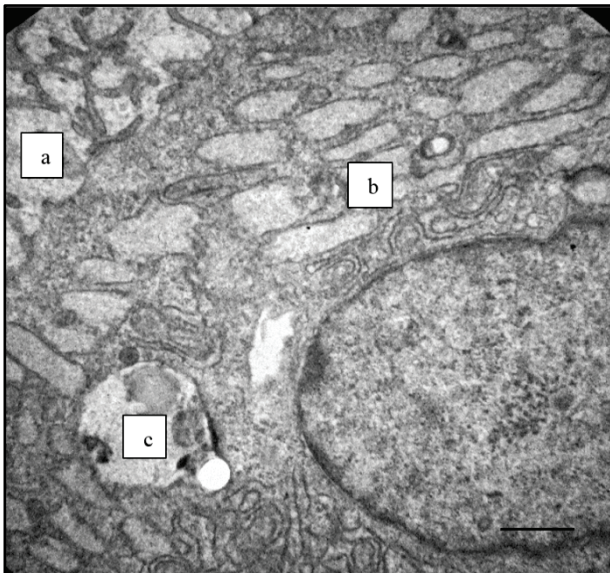


Fig. 8. Glandular cell of the proventriculus of a TVP-infected bird: rupture of intercellular contacts (a), sac-like lumens, surrounded by membranes of granular endoplasmic network (b); lysosome (c); scale bar 500 nm

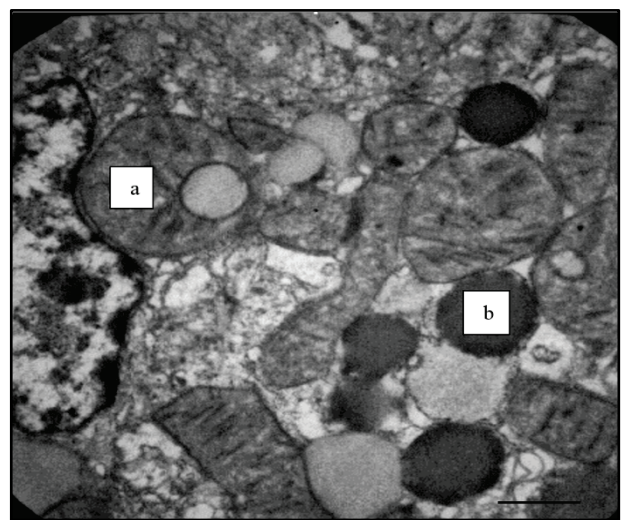


Fig. 10. Cytoplasm of secretory cell of the proventriculus of a TVP-infected bird: mitochondria (a); osmophilic secretory granules (b); scale bar 100 nm

There were cells where mitochondria contained optically transparent vacuoles, and their inner membrane was often in the state of complete destruction; some of them also had the outer membrane damaged as well, indicating irreversibility of the processes. In the cytoplasm of such oxynticopeptic cells, we also found osmophilic secretory granules, their characteristic morphological feature (Fig. 10). The development of active catabolic processes in cells also indicated concentrations of residuals of fragmented membrane structures. Furthermore, in cytoplasm of cells near such sites, pathogen virions localized freely (Fig. 11).

More often, we found desquamated secretory cells in which prevailed intensified destruction of organoids. Cytoplasm was filled with electron-transparent vacuoles, various fragments of residuals of the membranes of endoplasmic reticulum, swollen and deformed mitochondria with missing cristae. Growth of a large amount of optically transparent fields, various in shape and sizes, suggested elevated hydrophilicity of the cells (Fig. 12). Nuclei in such cells were mostly deformed, star-like or elongated shape, with large a bubbles, disturbed at some places. In karyoplasm, condensed chromatin dominated, which was localized as large conglomerates, and between its "boulders", there were diffusively scattered pathogen virions (Fig. 13).

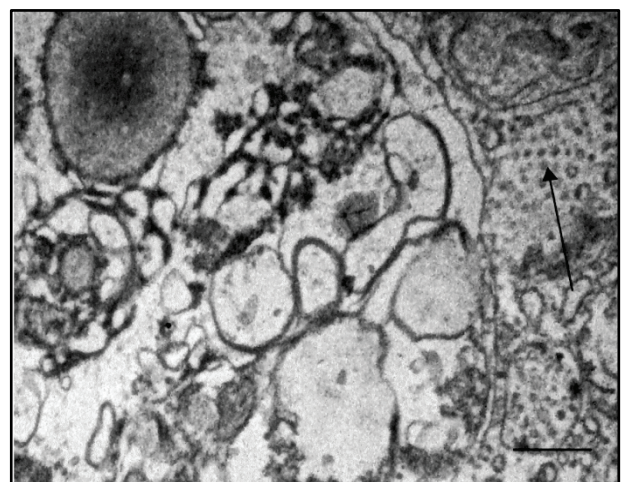


Fig. 11. Fragment of cytoplasm of desquamated glandular cell of the proventriculus of a TVP-infected bird; site of fragmented membrane structure and near arranged pathogen virions (indicated by arrow), scale bar 100 nm

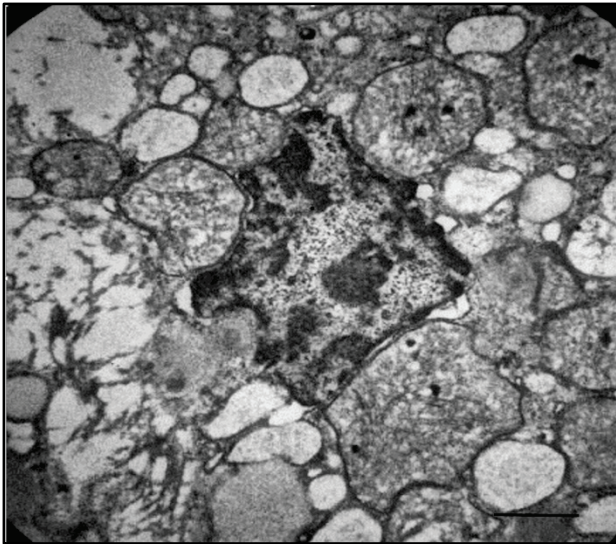


Fig. 12. Desquamated glandular cell of the proventriculus of a TVP-infected bird: cytoplasm is represented by electron-transparent vacuole, various fragments of residuals of membranes, swollen and deformed mitochondria with missing cristae; scale bar 500 nm

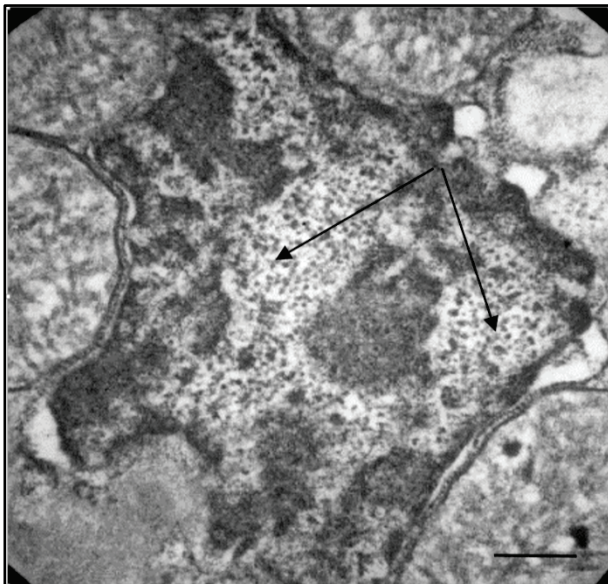


Fig. 13. Deformed nucleus of desquamated glandular cell of the proventriculus of a TVP-infected bird: the sac-like protrusions of the outer karyolemma membrane; pathogen virions were diffusely arranged in the karyoplasm between the boulders of condensed chromatin (indicated by arrows); scale bar 100 nm

Intense dying and shedding of glandular cells led to stacking of aggregated necrotic masses in the lumen of secretory sections. At the same time, we saw enlargement of lumens of the collective ducts and their being filled with eosinophilic detritus (Fig. 14). Manifesting dystrophic-necrotic processes in the tubular-alveolar structures were accompanied by recovery-compensative processes. However, the lost secretory epithelium was replaced not by non-glandular cells but the cells of excretory ducts. Metaplasia processes (transformation of secretory epithelium into cylindrical ductal epithelium) was accompanied by hyperplasia and hypertrophy. Alveolar walls of such region were distinguished against the general background, because they were much more thickened and their lumen was narrowed. Alveoli were lined by cells of cylindrical ductal epithelium densely adjacent to one another, with light-basophilic cytoplasm (Fig. 15). The observed changes in parenchyma were combined with active lymphoid infiltration of the interstitium. At the same time, mostly intraregional connective-tissue fibers of affected tubular-alveolar structures were loosely distributed, and their lumen was infiltrated by round-cell elements at

some places diffusely or as infiltration sites (Fig. 16). Their cellular infiltrate mostly contained lymphocytes, with occurrences of plasmatic cells, macrophages and heterophils. In some regions of the interstitium, multifocal lymphocyte sites have developed (Fig. 17).

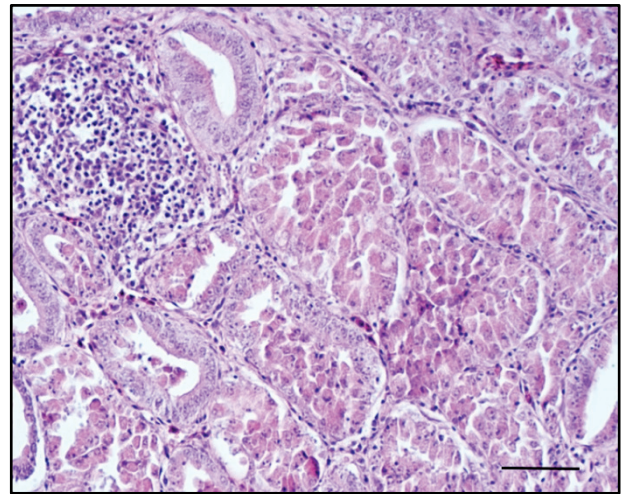


Fig. 14. Fragment of the area of the tubular-alveolar gland of the proventriculus of a TVP-infected bird: local interalveolar lymphoid infiltration; accumulation of desquamated glandular epithelium in the lumens of alveoli; transformation of the secretory epithelium into cylindrical ductal epithelium; hematoxylin and eosin; scale bar 50 μ m

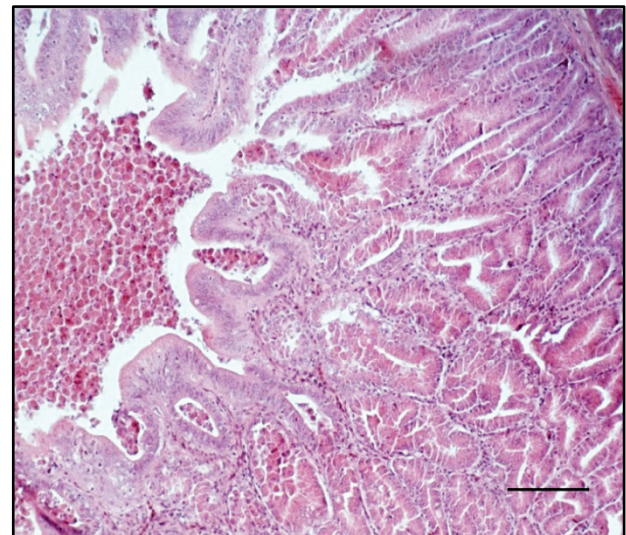


Fig. 15. Fragment of the area of the tubular-alveolar gland of a TVP-infected bird: eosinophilic detritus in the lumen of the alveoli and the excretory duct; lymphoid infiltration of the interstitium; hyperplasia and hypertrophy of the cylindrical epithelium of the duct; hematoxylin and eosin; scale bar 100 μ m

During histological study of the proventriculus of the TVP-infected birds, among comparatively undamaged tubular-alveolar structures, there were well distinguished areas with expressed destructive-necrotic processes in the secretory epithelium, and also areas in which the secretory epithelium was replaced by cylindrical epithelium of the ducts, which was always accompanied by lymphoid infiltration of interalveolar lumens. The observed changes indicate marked inflammatory process in proventriculus of the infected birds. Regardless of the period of disease during which the samples of proventriculus are gathered for a histological study, some alveoli will have alterations in secretory epithelium and others will have metaplasia and lymphoid infiltration. However, during the disease onset, the alteration manifests whereas the compensatory-adaptive processes intensify later.

Histologically, we determined progressing necrosis, desquamation of most of the glandular epithelium of tubular-alveolar glands and its re-

placement by cylindrical epithelium of excretory ducts. Ruination of the most functionally active secretory cells that produce pepsinogen and chlorides and their metaplasia on epithelial cells of ducts indicate steep decline in the secretory function of the proventriculus of the birds, expressed by drastic weakening of biochemical processes of feed digestion, decreased influx of nutrients into the organism, which reflected clinically in decrease in body-weight gains, body weight, and general anemic condition of the birds.

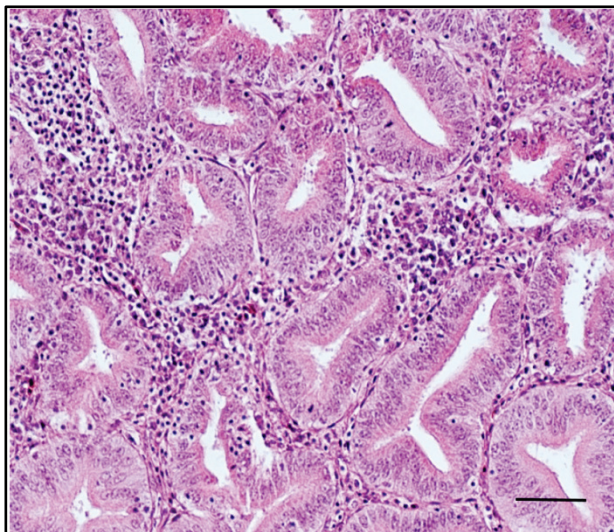


Fig. 16. Fragment of a region of the tubular-alveolar gland of the proventriculus of a TVP-infected bird: interalveolar lymphoid infiltration; replacement of the secretory epithelium by cylindrical ductal epithelium; hematoxylin and eosin; scale bar 50 μ m

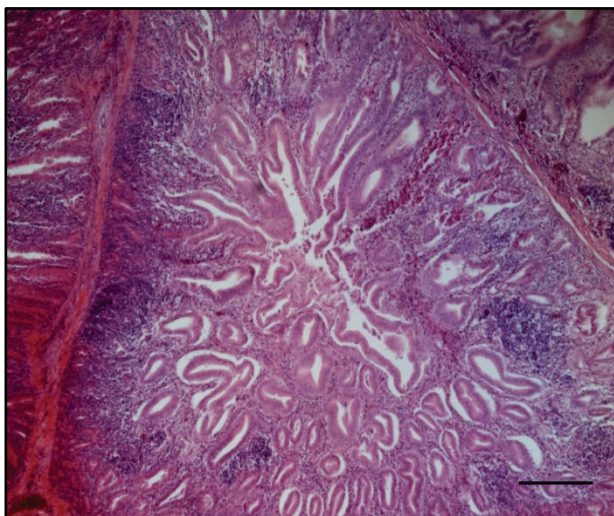


Fig. 17. Region of the proventriculus of a TVP-infected bird: lymphoid infiltration of the interalveolar membranes; hematoxylin and eosin; scale bar 100 μ m

Discussion

The glandular part of the stomach (proventriculus) of the birds is functionally equivalent to the mammals' stomach, and development of various pathological processes in its structure clinically manifests in digestion impairments, and this in turn leads to decreased productivity parameters of the birds. The pathohistological changes we observed in the proventriculus of the ill birds are consistent with the studies of foreign authors (Goodwin et al., 1996; Grau-Roma et al., 2010; Śmiałek et al., 2017), who reported the same processes during TVP. The causative agent of TVP is yet to be identified, which complicates the instrumental methods of disease diagnostics, though according to most researchers, the histological methods of

the study play the main role in diagnosing TVP. As reported (Guy et al., 2011; Hafner et al., 2013; Hauck et al., 2020), a complex of histological changes in the proventriculus of chickens should be taken into account, namely: massive necrosis of the secretory epithelium of the tubular-alveolar glands, its transformation into the ductal epithelium (metaplasia) and lymphoid infiltration of the interstitium. Detecting the specific histological changes in the proventriculus of sick poultry allowed us to diagnose transmissible viral proventriculitis in broiler chickens for the first time in Ukraine. It is interesting to note that in our study, no eosinophilic intranuclear inclusions in cells of necrotized epithelium were found, which were found in 22% of the TVP cases in other studies (Kouwenhoven et al., 1978; Goodwin et al., 1996; Guy et al., 2007; Grau-Roma et al., 2010).

As with the etiological factor, the main causative agent of the disease was for a long time considered the DNA-genome adenovirus (Kouwenhoven et al., 1978; Dormitorio et al., 2007). Based on the results of electron-microscopic and immune-histological studies, nuclei of cells of the proventriculus of sick birds were found to have adenovirus-like AdLV (R11/3), which – according to the data of reaction of indirect immunofluorescence and PCR – is distinct from well-known avian adenoviruses (Guy et al., 2005, 2007). Since 2011, most researchers have been insisting that TVP is caused by RNA-genome chicken proventricular necrotic virus (CPNV) of the *Birnaviridae* family, which was identified based on physical structure and partial genome sequence (Marguerie et al., 2011; Noiva et al., 2015; Grau-Roma et al., 2017, 2020). Moreover, we should note that adenoviruses and birnaviruses have a similar morphology. As of now, opinions of researchers regarding TVP etiopathogenesis vary and discussions continue. Scientists have put forward some hypotheses regarding effects of other viral, bacterial or fungal accompanying infections, which require further research.

According to the ultrastructural studies, we found presence of virions of the pathogen, diffusely located both in nuclei and cytoplasm of desquamated secretory cells, which correlates with the results of the studies by foreign authors (Goodwin et al., 1996; Guy et al., 2007, 2011). However, having analyzed the literature data and the results we obtained regarding ultrastructural changes in desquamated oxynticopeptic cells, we assume that the disease does indeed develop with participation of two pathogens. Our assumption is based on generally known data on replication of RNA and DNA-genome viruses in cells. As known, RNA-genome viruses usually replicate in cytoplasm and do not invade the nucleus of an infected cell, while DNA-genome viruses replicate in the nucleus, because they use RNA-polymerase II enzyme for transcription (Kalinina, 2016). The opinion we express confirms the hypothesis that clinical spontaneous cases of avian TVP develop with involvement of both RNA- and DNA-genome viruses, the individual significance and role of each of which in etiopathogenesis of the disease require in-depth researches.

Conclusions

Our histological studies of the proventriculus of the sick broiler chickens from three poultry farms in Lviv, Ivano-Frankivsk, and Volyn oblasts of Ukraine found necrotizing proventriculitis of lymphocytic type, with morphological features of progressing necrosis, desquamation of most secretory cells of the tubular-alveolar glands, and replacement of lost glandular cells by ductal cylindrical epithelium, combined with lymphoid infiltration of the interalveolar interstitium. The ultrastructural studies of nuclei and cytoplasm of desquamated secretory cells of the glandular part of the stomach of the sick chickens revealed presence of pathogen virions. Based on the detected histological changes in the proventriculus of sick poultry in Ukraine, we diagnosed transmissible viral proventriculitis.

The authors declare no conflict of interest regarding the authorship and publication of this study.

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