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Results of experimental infection of vaccinated and non-vaccinated ducklings with duck hepatitis virus

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SUMMARY
Duck virus hepatitis, a highly contagious disease occurring in ducklings, is currently reported in all countries with duck breeding industry. This infection is included in the list of notifiable diseases of the World Organization for Animal Health, restrictive measures are established and the regionalization of the country's territory is carried out in case of its occurrence. Timely vaccination of parent flocks in order to obtain immune offspring takes a priority place in the system of anti-epidemic measures. Due to active development of duck breeding industry and increasing number of backyard farms, the need for high-quality and effective prevention tools for this infectious disease is growing. To date, one domestic native vaccine produced by FGBI "ARRIAH" and registered in the Russian Federation is available in native form and is intended for parenteral use. In order to extend the vaccine's shelf life and for storage and transportation convenience, a live freeze-dried vaccine against duck virus hepatitis is being developed. The paper presents results of studying the pathogenesis of duck virus hepatitis and evaluating the efficacy and safety of the vaccine under development. Pathomorphological studies carried out post experimental infection indicate that duck hepatitis virus induces pathogenic effect not only in birds' digestive organs (the liver, in particular) but also causes degenerative changes in central and peripheral immune organs: in cloacal bursa, thymus and third eyelid gland, that may be manifested as deficiency of B- and T-cell immunity and requires further studies. It has been shown that in case of immunization of 3-day-old ducklings, the experimental vaccine induces antibody-mediated immune response, no harmful effect is produced on poultry if a tenfold dose is administered which indicates its safety, and the vaccine ensures protection against infection with a control virulent strain of hepatitis virus.

Keywords: duck virus hepatitis, pathogenesis, control strain, virus titre

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Результаты экспериментального заражения вакцинированных и невакцинированных утят вирусом гепатита

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РЕЗЮМЕ

В настоящее время такое высококонтагиозное заболевание, как вирусный гепатит утят, регистрируют во всех странах, где занимаются разведением уток. Данная инфекция включена в перечень нотифицируемых болезней Всемирной организации здравоохранения животных, при ее возникновении устанавливаются ограничительные мероприятия и проводится регионализация территории страны. В системе противоэпизоотических мероприятий ведущее место занимает своевременная вакцинация уток родительского стада с целью получения иммунного потомства. В условиях активного развития промышленного утководства и увеличения количества частных подворий растет необходимость в качественных и эффективных средствах профилактики данной инфекционной болезни. На территории Российской Федерации на сегодняшний день зарегистрирована одна отечественная вакцина производства ФГБУ «ВНИИЗЖ», которая выпускается в нативном виде и предназначена для парентерального применения. С целью увеличения срока годности препарата, а также для удобства его хранения и транспортировки ведутся работы по разработке вакцины против вирусного гепатита утят живой лиофилизированной. В статье представлены результаты изучения патогенеза вирусного гепатита утят и оценки эффективности и безвредности разрабатываемой вакцины. Проведенные после экспериментального заражения патоморфологические исследования свидетельствуют о том, что вирус гепатита утят оказывает патогенное действие не только на органы пищеварительной системы птиц, в частности на печень, но и вызывает дегенеративные изменения в центральных и периферических органах иммунной системы: в клоакальной бурсе, тимусе и железе третьего века, что может проявляться дефицитом В- и Т-клеточного звена иммунного ответа и требует дополнительного изучения. Показано, что при иммунизации утят в 3-суточном возрасте экспериментальный препарат индуцирует гуморальный иммунный ответ, введение десятикратной дозы не оказывает вредного воздействия на организм птиц и говорит о его безопасности, вакцина обеспечивает защиту от заражения контрольным вирулентным штаммом вируса гепатита.

Ключевые слова: вирусный гепатит утят, патогенез, контрольный штамм, титр вируса

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INTRODUCTION

Duck virus hepatitis type I (infectious duck hepatitis, DVH) is a highly contagious disease, occurring extremely acutely in ducklings up to 6 weeks of age and latently in ducks, which is characterized by liver lesions and high mortality of young birds. The disease causes significant economic damage to duck farms, in particular commercial holdings, since it causes death of 1–30-day-old ducklings (mortality rate is up to 95%) and a decrease in duck productivity. Convalescent ducklings are characterized by retarded growth and development, which results in partial loss of meat productivity and deterioration of breeding results. Duck virus hepatitis is registered in many European countries, in Southeast Asia, as well as in Egypt, the USA and Canada. The disease-caused damage increases due to the costs on restrictive measures that disrupt the economy of the farm, especially when the disease becomes persistent [1–4]. Duck virus hepatitis is included in the list of notifiable diseases of the World Animal Health Organization [5] and considered a quarantinable infection [6], subject to the regionalization carried out in the country [7].

The disease is caused by the RNA-virus belonging to the *Picornaviridae* family, the genus *Avihepatovirus* [8], which is classified into three types based on antigenic differences. Duck virus hepatitis type I is currently classified into 3 serotypes based on phylogenetic analysis and neutralization test results. Duck hepatitis virus serotype 1 is described in the literature as classic, it is spread all over the world and has the most significant impact on local poultry industry. On the contrary, DVH serotype 2 was detected only in Taiwan, and DVH serotype 3 was first registered in South Korea and recently identified in mainland China and Vietnam [9–12].

In natural conditions, ducklings of up to 40 days of age can be affected with viral hepatitis, but young birds are more likely to get diseased at the age of 1–30 days. Goslings of up to 10–12 days of age are also susceptible to DVH type I, when infected both naturally and artificially. Rapid development of age-related immunity is a characteristic feature of this infection, that is, older ducklings and adult ducks do not have clinical disease [3, 5]. The viral hepatitis cases and a sharp drop in egg production have been registered in laying duck populations in China since December 2016 [13]. The pathogenesis of DVH has not been studied enough. The DVH affects birds via different routes. Natural infection occurs horizontally, mainly through mucosa of digestive and respiratory organs [11, 14]. There is currently no evidence of vertical

transmission of the pathogen [15]. Penetrating into the body, the virus rapidly replicates and enters many organs hematogenically (primarily the liver and brain) [16]. The death of ducklings is caused by irreversible changes in the liver and other organs. As a rule, dead ducklings have a good body condition, though demonstrate signs of intoxication. As regards chronic viral hepatitis, lesions in the organs are of the same nature, but necrotic foci in the liver are more extensive [4, 17, 18].

Duck virus hepatitis is characterized by typical disease signs: anorexia, lethargy, reduced movement; long periods of ducklings sitting, loss of balance (ataxia). Occasionally diarrhea, rhinitis, conjunctivitis are observed. Spasms occur within 1–2 hours, less often 5–6 hours after the onset of neurological signs, while legs are stretched along the body, ducklings fall on their backs or on their sides with heads thrown back (opisthotonus), make paddling movements [14, 19]. Death occurs after several spasms. Complete recovery is rare, sometimes the disease becomes chronic, while birds lag behind in growth and development [2].

The necropsy of dead ducklings reveals that the most pronounced and characteristic changes are observed in the liver, which is noticeably enlarged in size, is ochre-yellow in color, the parenchyma is flabby, it easily collapses under pressure, in most cases the surface is strewn with hemorrhages varying from points to spots in shape. The gallbladder is generally full of bile. The cardiac muscle is dystrophic, resembles the appearance of boiled meat, the coronary vessels are blood-filled, an increased amount of serous fluid is often seen in the pericardial cavity [1, 2, 19].

Infected feed, water, litter, as well as contaminated poultry handling equipment are considered the pathogen's transmission factors. Such measures as quarantine and disinfection are used for control of DVH [8].

Due to the pathogen's variability and belonging to various serotypes, the DVH epidemic situation remains difficult in commercial duck breeding farms, but the disease can be effectively prevented by timely vaccination of adult ducks in order to obtain immune offspring [20]. Inactivated and live attenuated vaccines are used worldwide for specific disease prevention. China is the leader in the immunoprophylaxis of DVH using live vaccines [15]. The only domestic vaccine currently registered in the Russian Federation is the one produced by FGBI "ARRIAH" (Vladimir), it is available in native form and is intended for parenteral use. In order to extend the vaccine's shelf life, as well as for storage and transportation convenience, a live freeze-dried vaccine against DVH is currently being developed in the institution.

The aim of this research was to study the pathogenesis of DVH in experimental conditions, as well as to evaluate the efficacy and safety of the live freeze-dried vaccine against DVH.

MATERIALS AND METHODS

Tests to study the pathogenesis of duck virus hepatitis and the efficacy of the live freeze-dried vaccine against duck virus hepatitis were carried out in the Laboratory for Epizootology and Monitoring, the Center for Preclinical Tests and the Experimental and Biological Laboratory for Animal Studies (Animal Facility) of the FGBI "ARRIAH" (Vladimir).

One-three-day-old ducklings hatched within laboratory conditions from eggs obtained from OAO Plemproduktor "Zelenchuksky" (Karachay-Cherkess Republic) and showing anti-DVHV serum neutralizing antibody activity not higher than 3.0 $\log_2$, were used for testing.

Embryonated duck eggs were delivered to the laboratory at day 8 of incubation. The embryonated eggs were candled and quitters were discarded. Incubation was carried out at (37 ± 0.5) °C and a relative humidity of 80% before the ducklings were hatched.

The ducklings were kept in groups in isolated spacious cages with a mesh floor and individual lighting in a separate room in the animal facility. The birds were provided with water from automatic waterers, and were fed with dry mixed feed twice a day.

All experiments were carried out in strict accordance with the interstate standards for the maintenance and providing care of laboratory animals adopted by the Interstate Council for Standardization, Metrology and Certification, as well as in accordance with the requirements of the Directive of the European Parliament and Council of the European Union 2010/63/EU of September 22, 2010 on protection of animals used for scientific purposes.

Experimental live freeze-dried vaccine against duck virus hepatitis, Batch No. 010820, manufactured in the Laboratory for Epizootology and Monitoring of the FGBI "ARRIAH" according to standard procedure was used for vaccination of ducklings. The vaccine was produced based on duck hepatitis virus of the "VGNKI-K" production strain, obtained from the State Collection of Strains and Microorganisms of the FGBI "ARRIAH".

The ducklings were immunized at the age of 3 days. Before the vaccination procedure, a sterile saline solution was added to the vial with a freeze-dried product up to the initial volume of 3 cm^3. After resuspending, the vaccine was combined with the required volume of sterile saline solution at the rate of 100 cm^3 per 200 doses of vaccine. The vaccine, previously diluted with sterile saline solution at 1:10, was inoculated once into the femoral muscle group at 0.5 cm^3.

Blood sampling was conducted on days 7, 14 and 21 post vaccination by total bleeding for determination of serum antibody titer after vaccine administration. The titer was determined using indirect (passive) hemagglutination test (IHA test) in round-bottom plastic plates for serological assays using a 1% suspension of rooster red blood cells.

To prepare the erythrocyte antigen, the virus-containing material was centrifuged, then the sedimental liquid was mixed with a 1% suspension of rooster red blood cells and this mixture was kept in the refrigerator at a temperature of +4 °C for 24 hours for virus adsorption on the surface of erythrocytes.

After preparing all the necessary materials, double dilutions of the tested sera were made in round-bottomed plastic plates and the prepared erythrocyte antigen was added to the wells in equal volume. The test results were read in 30 minutes. The test result was considered positive in case of "umbrella formation" at the bottom of the well (hemagglutination). The test result was negative if red blood cells with adsorbed antigen sedimented to the bottom of the well and formed a "button".

To determine the safety of the live freeze-dried vaccine against duck virus hepatitis, the birds received a tenfold

Table 1
IHA antibody titre in sera of ducklings immunized with live freeze-dried vaccine against duck virus hepatitis ($n = 3$)

Day post vaccination	IHA antibody titre, $\log_2$
0	1.80 ± 0.20
7	5.00 ± 0.18
14	6.00 ± 0.40
21	5.60 ± 0.40

Table 2
Immunogenicity of live freeze-dried vaccine against duck virus hepatitis

Activity of duck hepatitis virus strain "Orekh", lg LD_{50}/0.5 cm^3	Number of ducklings that died/survived in the group	
	control	experimental
2.0	10/10	2/10

vaccine dose. For that, a sterile saline solution was introduced into the bottle with the freeze-dried vaccine to the initial volume. After resuspending, the vaccine was combined with the required volume of sterile saline solution at the rate of 100 cm^3 per 200 doses of vaccine. Undiluted vaccine (a tenfold dose) was administered to ducklings once in the femoral muscle group in a volume of 0.5 cm^3. Ducklings were observed for 10 days.

To determine the immunogenicity of the live freeze-dried vaccine against duck virus hepatitis, the birds were infected with the control virulent "Orekh" strain of the duck hepatitis virus, received from the State Collection of Strains and Microorganisms of the FGBI "ARRIAH". During the experiment, the ducklings were divided into experimental and control groups (10 birds in each group) and kept in isolated rooms. Three-day-old ducklings of the experimental group were immunized with a live freeze-dried vaccine against duck virus hepatitis at a dose of 0.5 cm^3. The control group ducklings were not immunized. After 7 days, birds of both groups were infected intramuscularly into the femoral muscle with the control virus strain "Orekh" with an activity of 2.0 lg LD_{50}/0.5 cm^3 in a volume of 0.5 cm^3. The birds were observed for 7 days.

Pathological and anatomical samples were collected from dead birds. The samples of internal organs were also taken from the ducklings of other experimental groups 7 days after vaccination for comparative histological examination and studying the effect of a tenfold immunizing dose on the body.

Histological examination was conducted on the basis of the Center for Preclinical Tests of the FGBI "ARRIAH".

Organ samples were fixed in 10% neutral formalin solution. To prepare tissue sections, the material was processed with the application of TLP-720 tissue processor (MtPoint™, Russia) and embedded in paraffin using ESD-2800 tissue embedding station (MtPoint™, Russia). Sections 5–8 μm thick were prepared at a rotary semi-automatic microtome RMD-3000 (MtPoint™, Russia), stained with hematoxylin and eosin using automatic linear stainer ALS-96 (MtPoint™, Russia).

The sections were examined using a MIKMED-6 microscope (AO "LOMO", Russia), and E3ISPM camera (China)

Fig. 1. Opisthotonus in ducklings that died after experimental infection with the control "Orekh" strain of duck hepatitis virus

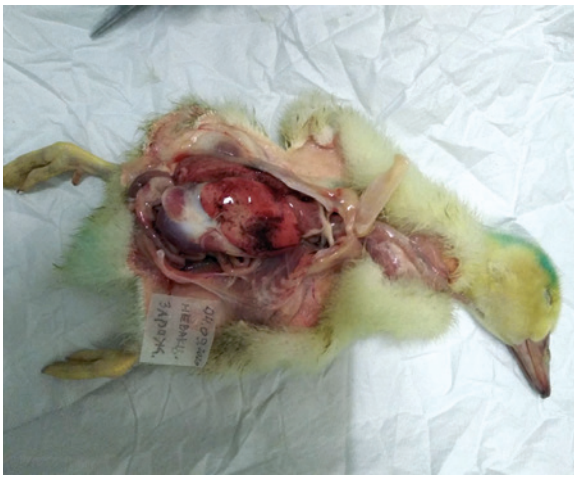

Fig. 2. Duckling that died after experimental infection with the control "Orekh" strain of duck hepatitis virus

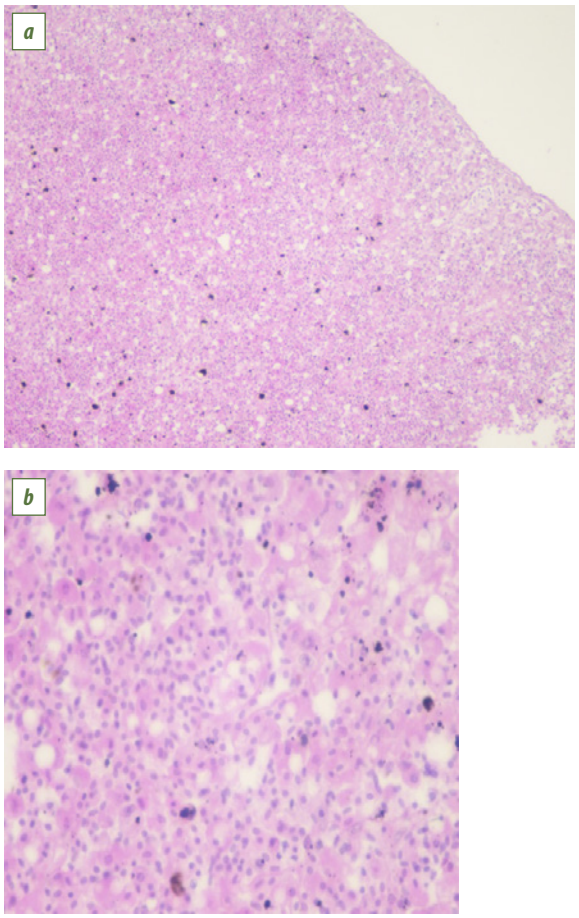

Fig. 3. The liver of a duckling that died after experimental infection with the control "Orekh" strain of duck hepatitis virus: a – 100× magnification; b – 400× magnification

and ToupView software (China) with 100× and 400× magnification were used for measurements and photographic documentation. A measuring scale of the camera was calibrated using OMP object micrometre for transmitted light (AO "LOMO", Russia).

RESULTS AND DISCUSSION

The test results for sera collected from vaccinated ducklings are presented in Table 1.

As it can be seen from the presented data, the experimental vaccine induced antibody-mediated immune response. Thus, the IHA antibody titer was (5.00 ± 0.18) $\log_2$ a week after vaccination. It reached the highest value of 6.0 $\log_2$ on day 14 after immunization and insignificantly decreased up to 0.4 $\log_2$ by day 21.

For the vaccine safety test, the birds were inoculated with a tenfold dose of the vaccine. During the 10-day observation period all ducklings remained alive and demonstrated no disease clinical signs. The necropsy did not reveal visible pathological and anatomical changes in the internal organs. This indicates that vaccination with increased vaccine doses does not have a harmful effect on ducklings and displays safety of the vaccine.

When the test for determination of the vaccine immunogenicity after infection of experimental and control group ducklings with the control pathogenic virus strain

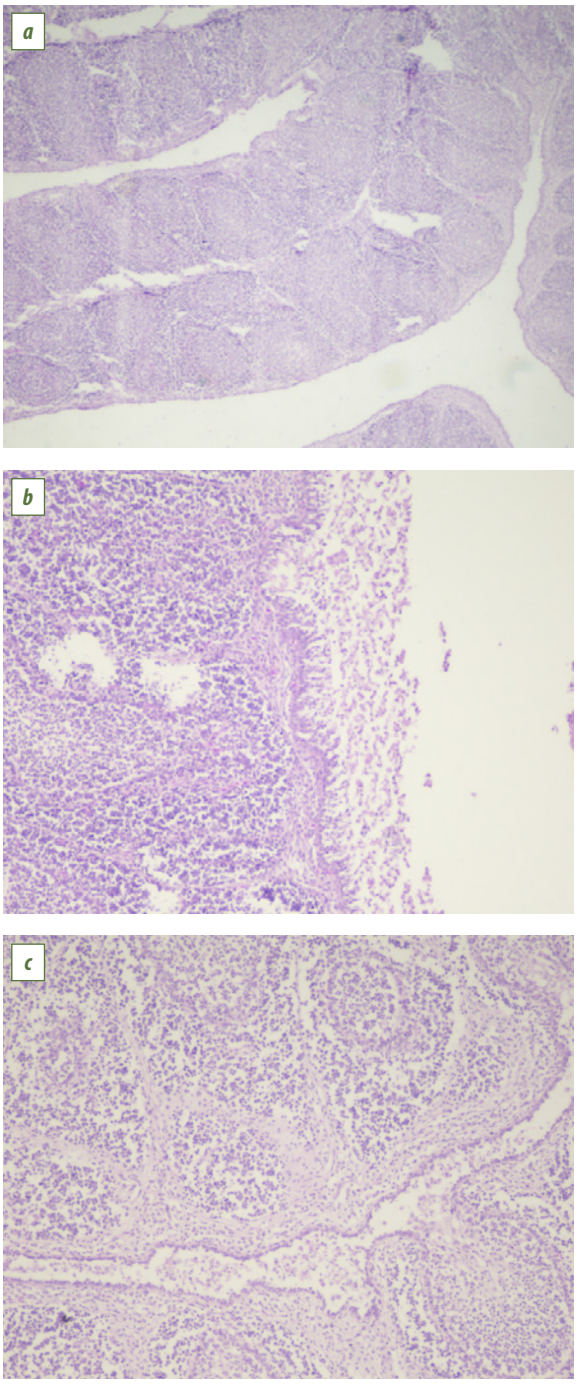

Fig. 4. Cloacal bursa of a duckling that died after experimental infection with the control "Orekh" strain of duck hepatitis virus: a – 40× magnification; b and c – 100× magnification

was conducted, the birds were monitored for 7 days. At the same time, the above-described clinical manifestations of the disease were noted, their development subsequently resulted in death.

As it can be seen from the results presented in Table 2, the experimental freeze-dried vaccine protected 80% of vaccinated ducklings from the disease in case of infection with the control virus strain "Orekh" with an activity of 2.0 lg LD_{50}/0.5 cm^3. All ducklings of the control group had typical clinical signs of the disease and died.

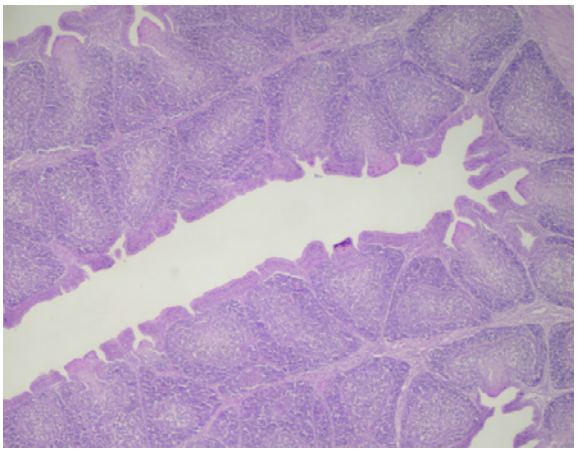

Fig. 5. Cloacal bursa of a vaccinated duckling after experimental infection with the control "Orekh" strain of duck hepatitis virus (40× magnification)

Thus, the post-vaccination antibody titre of 5.0 $\log_2$ protects 80% of ducklings from infection with a virulent strain of hepatitis virus.

Typical clinical signs were observed in non-vaccinated ducklings of the control group when the experiment aimed at studying pathogenesis of duck virus hepatitis after infection with the control pathogenic strain "Orekh" was conducted. The disease started acutely and progressed rapidly. In 24–48 hours after infection, with no change in feed and water consumption, the ducklings fell on their side or back, stretched their legs along the body, threw their heads back, which may be indicative for body intoxication due to the pathogenic effect of the virus on the liver and manifestation of a symptom complex in central nervous system damage. One – three hours after manifestation of clinical signs, ducklings died displaying opisthotonus (Fig. 1).

Visual examination of dead ducklings showed that they were well developed for their age. During the necropsy, the most typical and consistent of the detected disease signs were lesions in the liver that was enlarged, had a changed (yellowish-clay) color, was flabby and easily ruptured when pressed. Small point and spotty hemorrhages were observed on the surface and in the liver parenchyma (Fig. 2). A slight enlargement of the spleen and swelling of the pancreatic tissue were detected.

Histological examination of the liver of dead ducklings revealed diffuse hemorrhages; affected hepatic lobule structure; large-drop fatty dystrophy; macrophages, phagocyting apoptotic hepatocytes; hyperemia of blood vessels and sinusoidal capillaries; necrosis of some hepatocytes; hemosiderin inclusions (Fig. 3). Congestive hyperemia and edema were observed in the pancreas.

Total lymphocyte depopulation was detected in the bursa; thinning of the folds; necrosis of the epithelium of the folds; proliferation of interpholycular connective tissue; exudate in the lumen between the folds; swelling of the interstitial tissue; the boundaries between the cortical and cerebral substance were indistinguishable (Fig. 4).

Histological examination of cloacal bursa from vaccinated ducklings (after infection) showed its typical follicular structure with well-developed interfollicular stromal septa, corticular and cerebral zones (Fig. 5). The folds were well pronounced, numerous clusters of small and

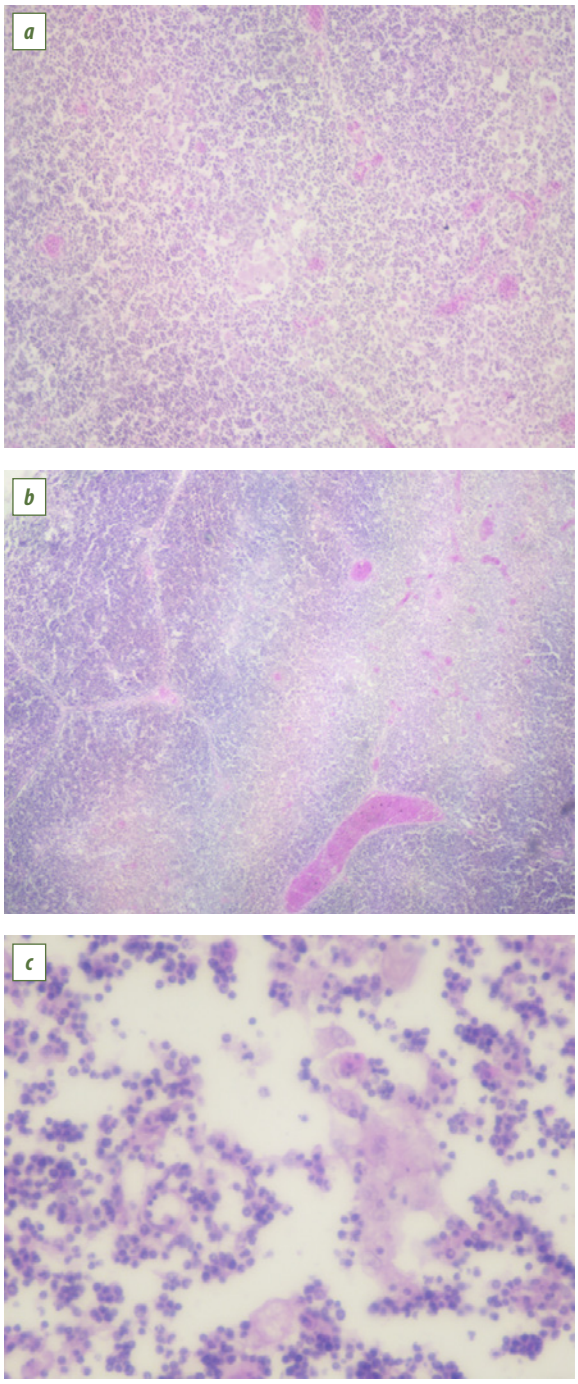

Fig. 6. Thymus of a duckling that died after experimental infection with the control "Orekh" strain of duck hepatitis virus: a and b – 100× magnification; c – 400× magnification

medium-sized lymphocytes were detected in the cortex. The delymphatization of the medulla was noted, which may be associated with immunocompetent responses to vaccination and subsequent infection. In some cases, the boundaries between the cortical and cerebral layer of the follicles became indistinguishable.

Histological examination of the thymus from dead ducklings revealed that the boundaries between the cortical and cerebral substance were not pronounced, the thymocytes were scattered. The formation of Ghassal bodies was noted in the medulla (Fig. 6).

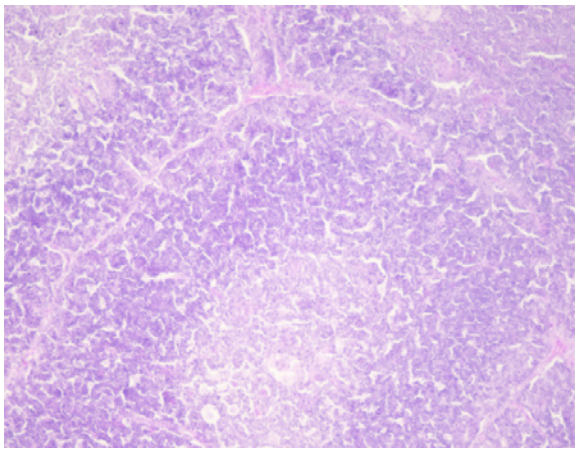

Fig. 7. Thymus of a vaccinated duckling after experimental infection with the control "Orekh" strain of duck hepatitis virus (40× magnification)

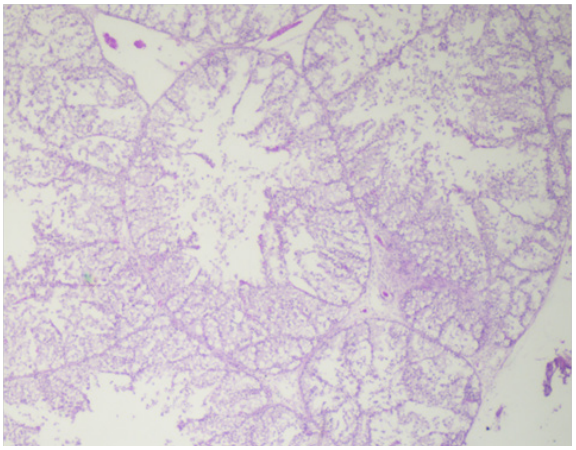

Fig. 8. Atrophy and cell depopulation in the third eyelid gland of a dead duckling

On the contrary, no deviations from the norm were observed in the thymus of vaccinated ducklings after infection, while the boundaries between the cortical and cerebral substance of the gland were well pronounced, the area of the cortical substance prevailed over the cerebral one (Fig. 7).

The examination of the third eyelid gland in ducklings infected with the pathogenic strain revealed atrophy and cell depopulation (Fig. 8, 9).

The necropsy results indicate that the duck hepatitis virus has a pathogenic effect not only on digestive organs, primarily the liver, but also causes degenerative changes in the central and peripheral organs of the immune system: in the cloacal bursa, thymus and third eyelid gland, which may be manifested as a deficiency of the B- and T-cell immunity response and requires additional studies.

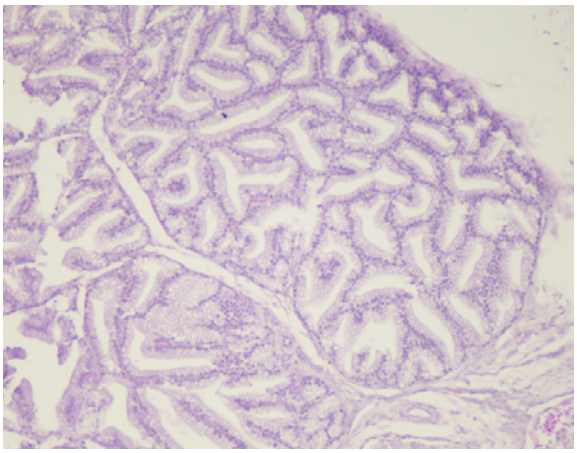

Fig. 9. Normal third eyelid gland

CONCLUSION

The conducted studies showed that live freeze-dried vaccine against duck virus hepatitis is safe for ducklings when administered in a tenfold dose and it induces protection from infection with a control virulent strain of the hepatitis virus.

Non-vaccinated poultry infected with the control virulent duck hepatitis virus demonstrate characteristic clinical signs, and pathologic anatomical changes found in the organs of dead birds are typical of acute duck hepatitis infection.

The study of pathomorphological changes in the organs of ducklings after infection with the hepatitis virus showed the virus's pathogenic effect on liver tissues and organs of the immune system, in particular on the thymus, cloacal bursa and third eyelid gland, which was demonstrated by accidental involution of the thymus and depopulation of lymphocytes in the cloacal bursa and third eyelid gland.

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