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PATHOLOGICAL FEATURES OF THE LUNGS AND LIVER OF PIGLETS UNDER CONDITIONS OF CONSTANT VACCINATION OF LIVESTOCK AGAINST CIRCOVIRUS INFECTION

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Abstract

The pathogenicity of PCV 2 in the body of vaccinated piglets was studied based on the results of pathomorphological changes in the lungs and liver of animals. The work was carried out on commercial piglets vaccinated with the vaccine Ingelvak CircoFLEX (Germany) against circovirus. The work used clinical, zootechnical, enzyme immunoassay and pathomorphological research methods. It has been established that under the conditions of ongoing vaccination of piglets against PCV2, 30.3% of piglets still do not have virus-neutralizing antibodies. The main reason for the culling of animals are circovirus diseases that have respiratory clinical signs, as well as signs of multisystem wasting syndrome, determining the safety of the livestock at the level of 68.05%, the average live weight of 1 head at the moment of its transfer for fattening is 40.44 ± 0.78 kg, and the average daily gain in live weight is 346.00 ± 9.18 g. At autopsy, sick piglets reveal an increase in the lungs and liver, and the signs of inflammation in them, as a result of circulatory disorders, damage to the lymphoid tissue, the development of dystrophic and necrotic changes. The results of the research suggest that in order to increase the efficiency of the formation of post-vaccination immunity, specific medical preparations can be used to stimulate the immune response of the body, as well as to enhance the resistance of the lymphoid tissue of the lungs and liver in animals.

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Introduction

In Russia, like in many countries of the world, pig breeding supplies most of the population's need for meat. The economic efficiency of the industry and the quality of the obtained products are directly related to the level of pigs' health, which depends not only on the technology of their feeding, managing, the genetic potential of their productivity, but also on the prevention of infectious and non-infectious pathologies in them [1–3].

Thus, among viral infections, the enterprises of the industry suffer the greatest economic damage not only from African swine fever (ASF), but also from circovirus diseases (CVD) [4, 5]. For example, in the USA the annual losses from the CVD amount to more than 600 million dollars, in Denmark — the annual losses make about 44 euros per sow.

Russia is no exception. Here from 80 to 100% of pig-breeding enterprises are "not safe" for being exposed to swine circovirus diseases. Chelyabinsk region, as a place of porcine farming, also suffers heavy losses from the porcine circovirus (PCV). This infection emerged and spread around Russia, as well as in other countries of the world, due to purchase of breeding pigs of foreign selection.

The main etiological agent of porcine circovirus diseases is porcine circovirus 2 (PCV2) [4], which not only suppresses the host immune system, but also enhances infection due to the replication of other pathogens (porcine reproductive and respiratory syndrome virus (PRRS), porcine parvovirus, swine influenza virus, swine fever virus) [6]. For example, when diagnosing pneumonia in pigs after weaning, the frequency of co-infection diagnosing is 42–85.4% of detected pneumonia cases [7]; in Chinese pig farms up to 52.4% of pigs are affected by PCV2 and PRRS, including service boars [8].

The complex of clinical signs in circovirus diseases associated with PCV2 includes "post-weaning multisystem wasting syndrome", respiratory diseases symptoms, enteritis, dermatitis, nephropathy, reproductive system issues, etc. [9–11].

At the same time, the most profound clinical signs of CVD are manifested in young pigs after their weaning, during the periods of their nursery and raising [10], thus causing the greatest rate of piglets culling. According to [4, 13, 14], the clinical manifestations of the infection develop due to the depletion of lymphoid tissue and lymphocytes, which proves the immunosuppressive functions of the PCV2, as well as its ability to infect and modulate the properties of a range of immune cells (macrophages, dendritic cells).

The main method of preventing pigs' circovirus diseases is vaccination. Among the prophylactic agents, PCV2 vaccines are the ones of the best-selling vaccines in pig farming [12, 15], as their use reduces mortality, decreases the culling rate of young animals, occurrence of concomitant diseases, and also increases the growth rate of the animals. After vaccination, virus-neutralizing antibodies appear in the body of pigs, and factors of humoral and cellular immunity are activated, thus determining the level of "immunological "protection" [14].

However, despite the ongoing vaccination, the PCV2 virus is constantly found among the vaccinated livestock. For example, in the United States in 2012 the virus was detected in 7.70–8.40% of vaccinated pigs [16], and in 2014–2016 in 11.30–29.00% already [17].

In Russia, the efficiency of vaccination ranges from 73.3–86.70% [18, 19] depending on the vaccine injected.

However, in recent years, studies have stated that before vaccination the virus was already present in the body of almost all animals, including sows [19, 20]. Therefore, at present, the decrease of the vaccination efficiency is caused not so much by the genetic variability of the virus, but to its constant circulation in industrial premises due to its high resistance to the environment.

According to [8, 12], the main reason of failing vaccination is the infection of animals before the vaccination. It happens when the healthy and infected pigs are kept together. Therefore, in the body of PCV2-vaccinated pigs, severe proliferative interstitial pneumonia and liver damage develop [21], that cause a mass mortality among the young animals as well as the decrease in pigs' growth rates and their meat quality.

To study the pathogenicity of PCV-2 in the body of piglets subjected to ongoing vaccination, we studied the specificity of pathomorphological changes in the lungs and liver of animals with a negative test for the presence of virus-neutralizing antibodies and which showed characteristic clinical signs of infection.

Materials and methods

Study design

The research part of the work was approved by the Bioethics Board of the South Ural State Agrarian University (Russia, Chelyabinsk region) and agreed upon with the veterinary service of LLC "Agrofirma Ariant" (Chelyabinsk region, Russia).

The design of the work provided for the formation of an experimental group of suckling piglets (n=3,618) at one of the commercial pig farm complexes of LLC "Agrofirma Ariant" (Chelyabinsk region, Russia).

Piglets on the 21st day of their life were vaccinated with the vaccine *Ingelvak CircoFLEX* (Germany) against circovirus in accordance with the vaccine manufacturer's recommendations.

At age of 23–24th days the piglets were weaned from their mothers and were transferred to a rearing shop, where they were distributed into group cages per 20–25 animals each.

All cages were identical in their design; every cage was equipped with automatic drinkers and feeders, which provided free access to water and feed.

The technology of feeding and managing the animals complied with the recommendations of Genesis.

Evaluation of the efficiency of post-vaccination immunity

Currently the following major methods are used to evaluate the results of vaccination and the efficiency of PCV2 vaccines:

1. Diagnostic method — the method based on determining the amount of virus-neutralizing antibodies.

To count the antibodies, blood was taken from the *vena cava cranialis* from 10% of the animals of the experimental group by random sampling at age of 60–70 days (40–50 days after vaccination). Blood samples were placed into a thermal container and were delivered within 3 hours after sampling to the Chelyabinsk branch of the Central Scientific and Methodological Veterinary Laboratory (Chelyabinsk, Russia). This lab determined the count of the specific antibodies to PCV2 with the help of the enzyme immunoassay method, using the CIRCOSerotest kits ("Vetbiokhim LLC", Russia) in accordance with the user's manual. The positive sample was considered valid if the coefficient of conjugate binding to blood serum antibodies exceeded 20%, and the negative one was less than 20%.

2. Production method — this method is based on the definition of the main production parameters: the safety of the livestock, average live weight (kg) of piglets at the end of the growing period and the average daily gain in live weight (g) for the nursery period.

The rate of livestock safety, i. e. livability (R_{saf}) for the nursery period was calculated by the following formula:

$$R_{saf} = 100\% - R_{mor}, \quad (1)$$

where:

R_{mor} — this is the rate of mortality and death of piglets in the experimental group.

For its calculation, the following formula was used

$$R_{mor} = \frac{P_{dead}}{P_{total}} \times 100, \quad (2)$$

where:

P_{dead} — is the number of dead and dead piglets (heads);

P_{total} — this is the total number of piglets in the experimental group (heads), 100 is the result conversion into a percentage.

The live weight of piglets was determined by the results of their individual weighing on the stationary livestock scales "Elton" (Russia). When calculating the average daily weight gains, we used data on live weight at the beginning and at the end of the nursery period.

Histological studies

The state of health in the experimental group of piglets was daily monitored by veterinary specialists of the pig farm. The piglets with clinical signs of CVD were transferred to the sanitary shop.

To obtain material for pathomorphological studies, from the sick animals we selected the sick piglets with a negative test for the presence of virus-neutralizing antibodies. The selected animals were subjected to euthanasia in the euthanasia unit of the agricultural company's recycling plant, and then their bodies were opened up to obtain biomaterial (lungs, liver).

Pieces of the liver and lungs, 1 cm^3 in size, were fixed with 10% formalin solution for 24 hours, washed with running water for 1 hour, and embedded in paraffin according to the following scheme: 1) sequential dehydration of samples in 70%, 80%, and 96% alcohol for 4 hours in each concentration; 2) overdrying in a mixture of alcohol and chloroform (1:1) for 1 hour, then in chloroform for 2 hours; 3) heating in a mixture of chloroform and paraffin for 1 hour at a temperature of 37 °C; 4) soaking with two volumes of paraffin at 56 °C for 45 minutes and making blocks.

Histological sections 5 μm thick were cut on the MS-2 sledge microtome (Russia). Before staining, the sections were deparaffinized in xylene for 2 minutes, then the xylene was removed with 96% ethanol for 2 minutes and washed with distilled water. For staining, a drop of hematoxylin was applied to the section for 2–3 minutes, washed off with water for 5–10 minutes, a drop of eosin was applied for 1 minute, washed off again with water, dehydrated in two volumes of 96% alcohol for 1 minute each, exposed to final dehydration in 100% alcohol for 1 minute and clarified in xylene for 2 minutes. After that the stained preparation was placed in a balm and covered with a coverslip. To detect lipids in the liver, formalin-fixed tissues were stained with sudan III (cerasine red) without embedding into paraffin. To do this, frozen sections were placed in an alcoholic dye solution for 15–20 minutes, rinsed in 50% alcohol, washed with distilled water, and embedded in glycerol for microscopy.

Sections were microscopically studied at various magnifications and photographed using a Leica DMRXA microscope (Germany) and a Leica DFC290 camera (Germany).

Statistical analysis

Statistical analysis considered the calculation of the mean value of the feature (X) and its standard error (Sx). The analysis was performed with the software "VERSIA". The signification of the differences was set at $p < 0.05$.

Results and discussion

Detection of specific antibodies to PCV2 after vaccination

Analysis of the results of enzyme immunoassay obtained from the Chelyabinsk branch of the Central Scientific and Methodological Veterinary Laboratory (Chelyabinsk, Russia) showed that in 10% of the samples from the experimental group, the share of negative samples amounted to 30.30%. Consequently, no post-vaccination immunity was formed in these animals after the vaccination, which accordingly created the basis for the circulation and reproduction of the virus in their body.

Clinical signs

All piglets euthanized by us were classified as infected with PCV-2, as they had not only a negative test for the presence of virus-neutralizing antibodies after vaccination, but showed the following clinical signs as well:

1. Oppressed behavior, food refusal, weight loss, inanition of the body, pale skin.

2. Shortness of breath, cough, nasal discharge, mucous membranes were either pale or icteric, fever. Some animals featured nervous issues in the form of tremors and paralysis. Inguinal superficial lymph nodes were enlarged.

The combination of these clinical signs proved that the piglets suffered from multisystem wasting syndrome and respiratory diseases.

Parameters of livestock safety and growth of piglets

The safety of piglets in the experimental group during the nursery period was equal to 68.05% (Figure 1). When comparing this parameter with the number of negative samples obtained during detection of specific post-vaccination antibodies to PCV2, it can be stated that the main reason for the culling of the young animals of the sampled group was the development of circovirus infection, as the basis for CVD formation.

The main zootechnical parameters, characterizing the growth rate of piglets during the nursery period, indicated that the average live weight (LW) of 1 head at the moment of its transfer for fattening was 40.44 ± 0.78 kg, and the average daily live weight gain for the considered period was 346.00 ± 9.18 g (Figure 1).

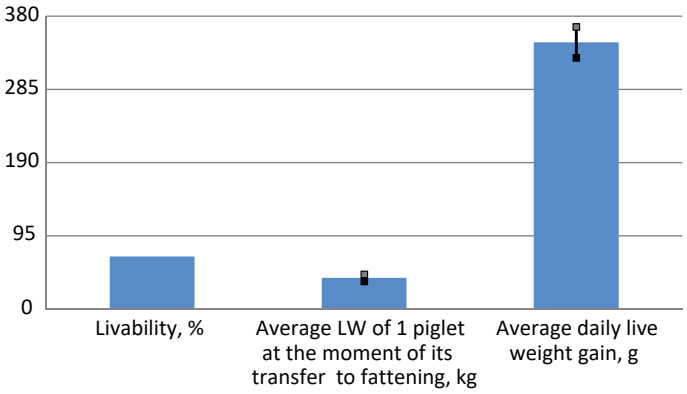

Figure 1. Indicators of safety and productivity of animals

Macroscopic features of the piglets' lungs and liver

At autopsy, the piglets showed an increase in lungs size. They were dark red and had blunt edges; foamy fluid was accumulated in the trachea and bronchial lumens. The texture of the organ was dense or rubberish, but with a well-defined lobation. At the same time, the spaces between the lobules were expanded and filled with a clear liquid. Mucous liquid was released from the small bronchi when pressed. The mucous membrane of the large bronchi was reddened, swollen and covered with plentiful mucus.

Macroscopic evaluation of the liver found an increase in its size and the blunt edges. The parenchyma of the organ was bulging on the incision and the edges of the incision did not converge anymore; the texture was flaccid. The surface of the organ featured a grayish tint, in some places there were yellow spots, the lobulation pattern of was blurred. The liver was easily torn when pressed.

Histological features of the lungs

Histological examination of the lungs in all fields of view showed a pronounced vascular congestion in all veins of any calibers with a clear picture of erythrostasis and erythrocyte thrombi in the capillaries (Figure 2A, 2D). Alternating large areas of dystelectasis and atelectasis, small foci of acute

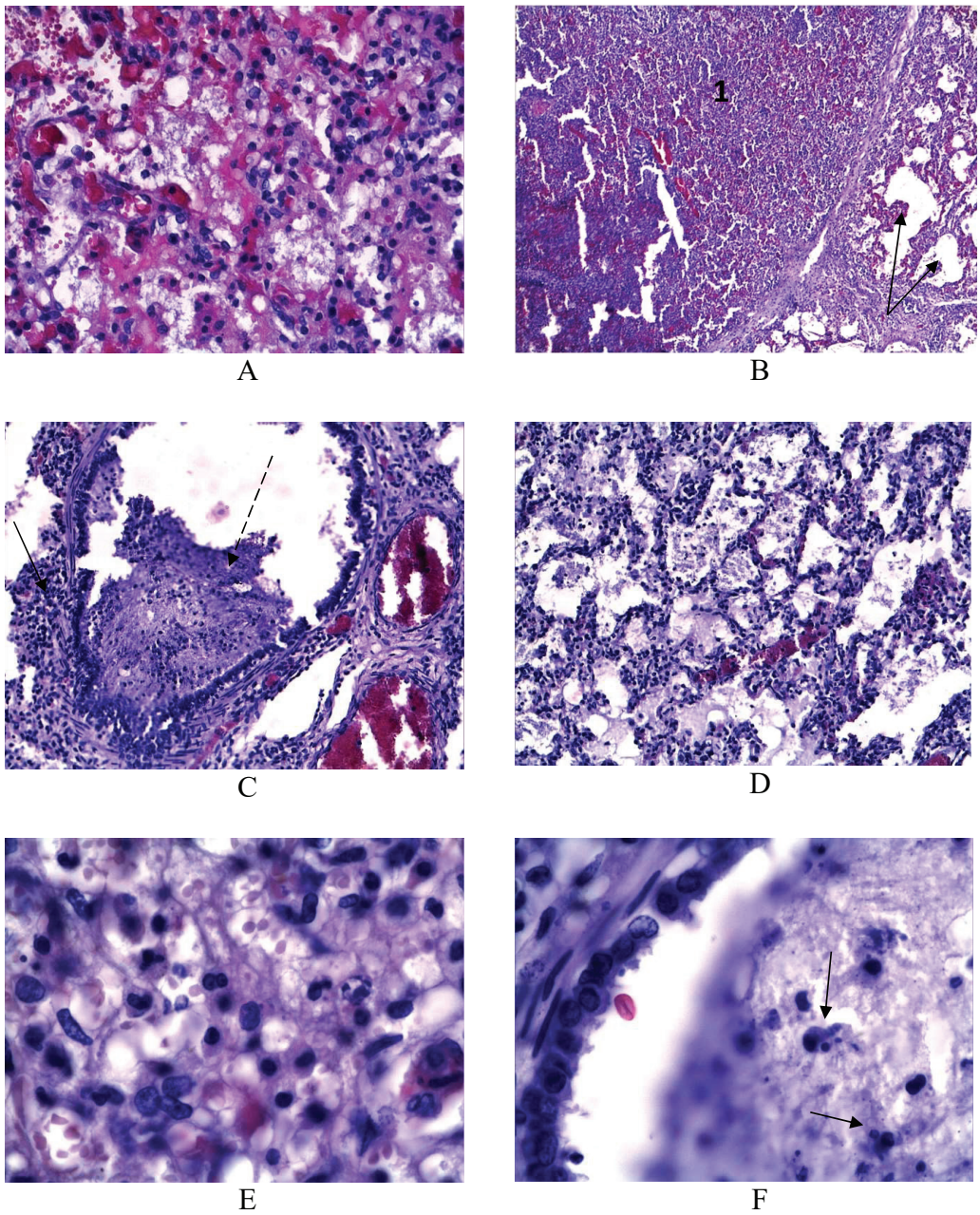

Figure 2. Morphological changes in the lung:

A — paretic plethora of vessels of all calibers with a picture of erythrostasis and erythrocyte thrombi in the capillaries, magnification x200; B — extensive area of atelectasis (1) and small foci of acute emphysema (arrows), magnification x50; C — neutrophil-lymphocytic infiltration of the peribronchial connective tissue (arrow); in the lumen of the bronchus — layers of desquamated epithelium, fine-grained structureless masses with an admixture of cells of the granulocytic series (dashed arrow), UVX200; D — focus of serous pneumonia, uv.x200; E — detail of the picture "D": in the cellular composition of the exudate, lymphocytes, neutrophilic leukocytes, erythrocytes and macrophages are visible, magnification x1000, MI; F — rounded inclusions (arrows) in the cytoplasm of macrophages in the lumen of the bronchus, x1000.
Stained with hematoxylin and eosin.

emphysema (Figure 2B) were found. Bronchial walls of all calibers were infiltrated with granulocytic cells, predominantly neutrophilic leukocytes and lymphocytes with small amount of eosinophilic leukocytes (Figure 2C). The lumen of the bronchi is uneven due to the lungs walls folding. Bronchial epithelium with a large amount of amorphous mucous masses on the surface was partially desquamated. The lumen of the bronchi was filled with layers of desquamated epithelium, with fine-grained, structureless masses with an admixture of a moderate amount of lymphocytes and neutrophilic leukocytes (Figure 2C). The lumen of the alveolar groups was filled with exudate consisting of fine-grained structureless masses and a small amount of granulocytic cells with an admixture of desquamated alveolar epithelial cells, macrophages and erythrocytes (Figure 2D, 2E). At high magnification, small, round, optically dense basophilic inclusions (most likely of a viral nature) were detected in the cytoplasm of macrophages (Figure 2F).

The walls of the alveoli were thickened due to neutrophil-lymphocytic infiltration; the capillaries here were sharply congested, with a peculiar pattern of widespread erythrocyte thrombosis (Figure 2A, 2D).

Histological features of the liver

When examining the microscopic picture of the liver in all fields of view — paretic venous and capillary plethora. The lobules are clearly visible. Cords discomplexation is caused by expansion of the pericapillary spaces (Figure 3A). The walls of the portal tracts are sharply edematous, defibular in structure, with dense neutrophil-lymphocytic infiltration (Figure 3B). Infiltrate cells migrate through the pericapillary spaces deep into the parenchyma (Figure 3D). The central veins are dilated, their walls are swollen, edematous, in the pericapillary spaces, large amounts of loosely piled, and clearly contoured erythrocytes are detected with an admixture of evenly distributed white blood cells well-visible against their background (Figure 3C). Hepatocytes are in state of severe protein vacuolar dystrophy, up to necrobiosis. Their nuclei are of different sizes — from small hyperchromic to enlightened bubble-shaped nuclei. When stained with sudan III, all preparations showed a small amount of fat droplets in the cytoplasm of hepatocytes under the organ capsule (Figure 3E, 3F).

The combination of pathomorphological changes in the lungs and liver of circovirus-infected piglets made it possible to classify these organs as PCV2 targets, since their lymphoid tissue played an important role in formation immune processes nature and direction.

Circovirus diseases are widespread in pigs farming in almost all countries of the world, and Russia is no exception. Meanwhile vaccination is the main way of preventing viral infection [12, 15].

However, continuous ongoing vaccination does not provide complete immune protection of piglets against PCV2, thus leading to losses in the livestock, as well as to replication and transmission of the virus. This was evidenced by both the number of negative samples (30.30%) in the experimental group when virus-neutralizing antibodies were detected in the body of vaccinated animals, by the level of animals livability (68.05%) and by zootechnical parameters (average live weight of a piglet at the moment of its transfer for fattening, its average daily gains in live weight).

Accordingly, the combination of these parameters influenced the economic performance of the enterprise and its profitability. According to [22, 23], one of the reasons for this trend is that PCV2 vaccination can induce the emergence of virus variants that resist the vaccine. In result the overall prevalence of PCV2 positive herds becomes unchanged. At the same time, the use of monovalent PCV2 vaccines can provide protection only against homologous infection, since these vaccines do not provide complete cross protection against other genotypes of PCV2 [24].

However, for vaccination the pig farming company, being an experimental venue, used the vaccine *Ingelvak CircoFLEX* (Germany), which is effective against all strains of PCV-2: PCV-2a, PCV-2b, PCV-2c, PCV-2d. Therefore, it is logical to suggest that a certain part of the piglets before vaccination was either seropositive [19, 20], or maternally derived antibodies circulating in their body, did not allow the formation of the required level of immune protection against the infection [22].

The formation of a healthy livestock of animals and the success of getting a full-fledged immune response when using a vaccine depends on the state of cells and tissues at the possible inlets of infection and the liver, as the liver serves as biological laboratory responsible not only for the disinfection of metabolic products, but also the formation of immunity [25–27].

According to [28] the most common clinical symptoms of circovirus infection in porcine, especially in nursery piglets, are the signs of respiratory diseases, as well as of digestive organs. Therefore, we chose the lungs and liver as the priority internal organs that play a crucial role both in the vital processes of the pigs in the state of the "conditional norm" and in the formation of their immune resistance to the virus.

According to [15], PCV2 in animals affects lymphoid tissue. In the lungs the lymphoid tissue is associated with the bronchi, ensuring the flow of immune reactions.

Our studies have found that in the lungs of circovirus infected pigs, the processes of circulatory disorders and inflammation were observed, which processes were associated with catarrhal inflammation and impaired microcirculation of the body's immunocompetent cells. In the cells of the first line of defense — i. e. lung macrophages — microscopic analysis found the included bodies of basophilic color, which were the result of the assembly and accumulation of circovirus virions. Our observations are consistent with the data [29] as those bodies are a consequence of viremia [30]. Meanwhile it was noted [25] that alveolar epithelial cells in the local area of the lung are the most sensitive to the virus, and start the congestive processes at the local edge of the lung lobule.

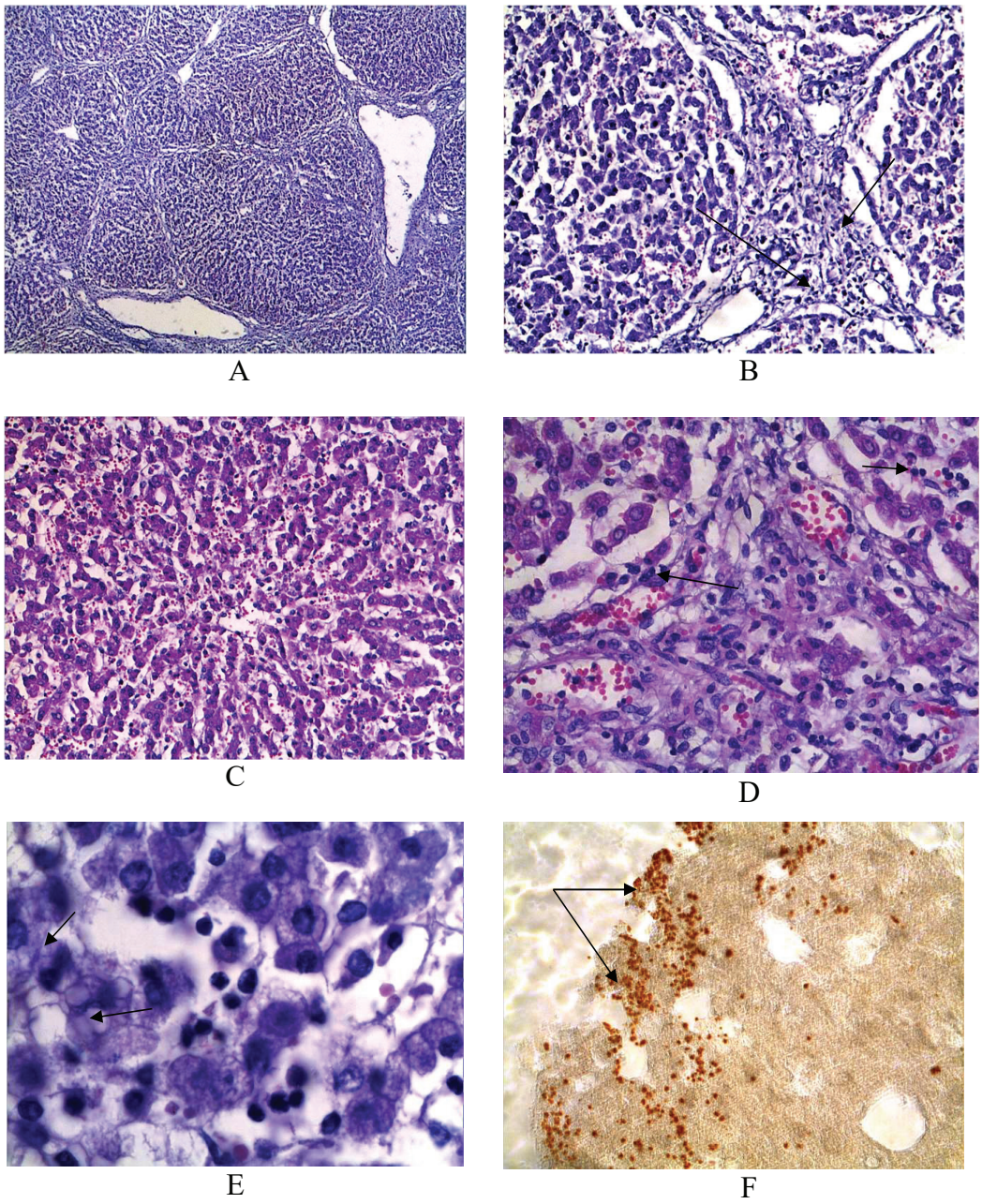

Figure 3. Morphological changes in the liver:
A — a distinct lobed structure, discomplexation of the hepatic cords, uv.x50; B — edema of the portal tract wall with infiltration by cells of the granulocytic series (arrows), magnification x200; C — the center of the lobule: swelling of the pericapillary spaces, a sharp plethora of sinusoids, magnification x200; D — cells of the neutrophilic-lymphocytic infiltrate spread through the expanded pericapillary spaces deep into the parenchyma (arrows), magnification x400; E — severe proteinaceous and hydropic (arrows) dystrophy and polymorphism of hepatocyte nuclei, magnification x1000; F — small accumulations of fat droplets (arrows) in the cytoplasm of hepatocytes under the liver capsule, magnification x100.
Staining: A-E: hematoxylin — eosin, F — sudan III.

The immune response is impossible to form without maintaining the homeostasis of the animal body with the liver [31], as this organ consists not only of hepatocytes but of immune system cells also (for example, macrophages — Kupffer cells, endothelial cells, pit cells, polymorphonuclear leukocytes, T- lymphocytes and B-lymphocytes). Moreover, the liver regulates the level of antigenic load on the animals' organism [32].

According to our studies, the liver of circovirus infected pigs features the processes of circulatory disorders and severe protein-fat degeneration up to hepatocyte necrosis. According to [33] liver damage is a frequent finding in microscopic studies in cases of porcine circovirus infection, and hepatocytes are the target cells for infection and virus replication [11, 34].

Conclusion

According to the results of these studies it was found that under the conditions of ongoing PCV2 vaccination of piglets with the vaccine *Ingelvak CircoFLEX* (Germany), no virus-neutralizing antibodies were detected in 30.3% of the piglets. Meanwhile, some individual piglets with clinical signs of multisystem wasting syndrome and respiratory diseases were constantly found.

This very symptomatic complex is the main reason for rejecting of animals, which reduces the safety of

livestock in the experimental group down to 68.05% and makes the average live weight of 1 piglet around 40.44 ± 0.78 kg (weighed at the moment of transfer to fattening stage), the average daily live weight gain is generally 346.00 ± 9.18 g.

At autopsy, sick piglets showed increased lungs and liver, and the signs of inflammation in these organs. Those problems were caused by circulatory disorders, damages to the lymphoid tissue, and development of dystrophic and necrotic changes.

The results of this study suggest that in order to increase the efficiency of post-vaccination immunity formation, specific medical preparations can be used to stimulate the immune response of an animal body, as well as to stimulate the resistance of the lymphoid tissue of the animal lungs and liver.

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