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Original research

Immune response modulation and gastric ulcer progress are associated with PCV-2 infection in farming pigs

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Abstract. Gastric ulcer in pigs is a common complication of livestock feeding worldwide. Ulcers lead to reduced feed consumption, decreased daily weight gain, and even sudden death, resulting in certain economic losses. Ulceration in the non-glandular part of a pig's stomach is a disease of complex etiology, where disruptions in feed intake, particle size, nutrient content, infections, and stress play important roles. Ulceration of the non-glandular gastric mucosa occurs in pigs of any age, but the highest level of ulcers is observed in pigs aged 3-6 months. The exact cause of ulcer formation remains unclear. Recently, porcine circovirus type 2 (PCV2) has been considered as one of the potential causative factors of gastric ulcers in pigs. The aim of the study was to investigate molecular markers of gastric epithelium damage and the induction of gastric ulcers under the conditions of PCV2 viral infection. The research was conducted at the Pig Complex and the Research Center for Biosafety and Environmental Control of Agricultural Resources at Dnipro State Agrarian and Economic University "Biosafety-center" (Dnipro city). Histological, immunohistochemical, and PCR studies were carried out on stomach tissue samples with manifestations of ulcers. The results of histological studies revealed several findings in the fundal part of the stomachs. These included erosive gastritis, lymphadenitis, and inflammation in mesenteric lymph nodes. Additionally, pathological-histological signs of recurrent rhomboid-shaped cardiac ulcers were observed. These ulcers had an average size of 5×4.9 cm and a depth of approximately 2/3 of the thickness of the muscular layer. Furthermore, purulent-necrotic fibrinoid detritus was evident at the edges of the ulcers. PCR analysis confirmed the presence of PCV2 infection in all animals with ulcers. At the same time, PCV2 was not detected in the control group. In the stomach tissue of animals with ulcers, a statistically significant decrease in interferon-alpha content ($P < 0.05$) relative to the control was determined. The decrease in interferon production in damaged areas of the stomach indicates suppression of innate immunity. The obtained results indicate that PCV2 infection, along with weakened immune response, can be an important factor in initiating ulcerative disease and functional disturbances of the intestinal system in pigs.

Keywords: gastric ulcer; porcine circovirus infection; immune response; cytokine production.

Модуляція імунної відповіді у розвитку виразки шлунку свиней асоційованої з PCV-2 інфекцією

Анотація. Виразка шлунку у свиней є поширеним ускладненням промислових тварин у всьому світі. Виразка спричиняє зменшення споживання корму, зниження щоденного приросту ваги та, навіть, раптову смерть, що призводить до певних економічних втрат. Виразка в незалозистій ділянці шлунка свиней є захворюванням складної етіології, в якій важливу роль відіграють переривання споживання корму, розмір часток корму, вміст поживних речовин, інфекції та стрес. Виразка незалозистої слизової оболонки шлунку трапляється у свиней будь-якого віку, але найвищий рівень виразки спостерігається у свині 3-6 місячного віку. Точна причина виникнення виразки залишається не зовсім зрозумілою. Останнім часом цирковірус типу 2 (PCV2) розглядається як один з потенційних причинних факторів виразки шлунку свиней. Метою роботи було дослідження молекулярних маркерів ушкодження епітелію шлунка, індукція виразки шлунку за умов вірусної інфекції PCV2. Дослідження проведені на базі свинокомплексу та науково-дослідному центрі біобезпеки та екологічного контролю ресурсів АПК ДДАЕУ «Biosafety-center» (м. Дніпро). Гістологічні, імунохімічні та ПЛР дослідження проводили у зразках тканини шлунку з проявами виразки. При аналізі гістологічних досліджень було виявлено, що в донній частині шлунків спостерігається ерозивний гастрит, лімфаденіт, запалення у мезентеричних лімфатичних вузлах та патолого-гістологічні ознаки рецидивуючої кардіальної виразки ромбоподібної форми розміром в середньому 5×4,9 см глибина яких була приблизно 2/3 товщини м'язової оболонки з гнійно – некротичним фібріноїдним детритом по краях виразки. ПЛР аналіз показав наявність PCV2 інфекції у всіх тварин з виразкою. В групі контролю PCV2 не було виявлено. У тканині шлунку тварин з виразкою було визначено статистично значуще зниження вмісту інтерферону-α ($P < 0,05$) відносно контролю. Зниження продукції інтерферону в ушкоджених ділянках шлунку свідчить про пригнічення уродженого імунітету. Отримані результати показали, що PCV2 інфекція разом з послабленням імунної відповіді може бути важливим факторам ініціації виразкової хвороби та функціональних порушень інтестинальної системи свиней.

Ключові слова: виразка шлунку; цирковірусна інфекція свиней; імунна відповідь; продукція цитокінів.

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Introduction

Modern intensive pig farming methods have both positive and negative aspects that affect the efficiency of pig production. A large number of factors, including high stocking density, farm caging of animals leading to hypodynamia, early weaning, and the use of specialized concentrated feeds, can disrupt the function of the gastrointestinal tract, overall body condition, metabolic processes, and natural resistance levels (Alarcon et al., 2013; Wang et al., 2020). In most developed countries with intensive pig farming, gastric ulcers in pigs are not specific to breed or gender while they are widespread and account for up to 93% of all gastrointestinal diseases in pigs (Park et al., 2020; Cybulski et al., 2021). Gastric ulcers are a cause of animal welfare issues, with up to 15% of all affected pigs leading to death (Levchenko et al., 2005).

The stomach of pigs has a unique anatomical structure, and erosions and ulcers in adult pigs are mainly localized in the esophagogastric region, which is a non-glandular part of the gastric mucosa (Marchini et al., 2017). The initiation of gastric ulcers in pigs is multifactorial, primarily associated with pig feeding technology. This includes inconsistent feeding processes, disrupted drinking regimes, the use of finely ground feed particles smaller than 400 micrometers, as well as feed with low fiber and protein content and high levels of unsaturated fats. An imbalance in diets regarding the content of micro- and macro-elements, particularly high copper concentration and low zinc content in feed and water, plays an important role in initiating gastric ulcers (Phillipson, 2008; Jorgensen, 2014; Zhang et al., 2018).

Secondly, social stress factors contribute to the initiation of gastric ulcers in pigs. These include constant regrouping of pigs, leading to changes in group composition, prolonged transportation, overcrowding, disruptions in microclimate within facilities, and gastrointestinal parasitic infestations due to inadequate parasite control measures (Segalés, 2012; Choi, 2018; Figueras-Gourgues et al., 2019). Detrimental changes in the animals' immune status occur against the backdrop of a significant increase in negative impacts on the pig's organism and a sharp rise in the quantity and intensity of human activity factors, such as untimely parasite control measures and ineffective vaccination (Zhai et al., 2016; Lee et al., 2021).

One of the main reasons for the occurrence and development of gastric ulcers in pigs is infectious factors. According to numerous modern studies, it has been shown that the primary role in the etiology and pathogenesis of gastric ulcer disease belongs to porcine circovirus type 2 (PCV2) (Masiuk et al., 2016; Dunkelberger et al., 2016; Franzo, 2020; Noh et al., 2022). Porcine circovirus type 2 belongs to the family Circoviridae, genus Circovirus, and possesses a single-stranded circular DNA (ssDNA) genome ranging in length from 1767 to 2020 nucleotides. It exhibits a high mutation frequency and is pathogenic to pigs (Dvorak et al., 2018; Opriessnig et al., 2020).

Due to various etiological factors contributing to gastric ulcers and the diverse forms of manifestation, circovirus infection is widely spread among industrial pig populations worldwide. It leads to significant economic losses due to reduced growth rates of pigs and high mortality among fattening animals. PCV2 is associated with several disease complexes and is the primary infectious agent in porcine multisystemic wasting syndrome (PMWS). One of the diagnostic indicators for PMWS is the presence of nucleic acids and high levels of PCV2 antigen (Yang et al., 2020; Saporiti et al., 2021; Zhang et al., 2021). Pathomorphological changes in pigs infected with PCV2 occur moderately and in stages due to the simultaneous activation of complex disease resistance mechanisms, resulting in the formation of a spectrum of reactive and pathological processes (Zhang et al., 2021).

The primary target of PCV2 infection in the animal organism is the immune system, which in pigs, like in other mammalian species, is structurally and functionally well-developed. PCV2 replication in immune system cells leads to their destruction and the development of an immunodeficient state, which enhances infection and replication of other pathogens. However, the mechanisms of organic damage development in stomach tissue remain unclear (Vincent et al., 2007; Zhang et al., 2020; Wu et al., 2021).

The aim of the study was to find out the role of molecular markers of gastric epithelium damage and the induction of gastric ulcers caused with porcine circovirus type 2 (PCV2) infection.

Materials and methods

The study was conducted using animals from a farrow-to-fattening pig production system. Laboratory research was carried out at the Biosafety Center, a research center for environmental control and biosafety of agricultural resources, located in Dnipro city. Animals aged 17 weeks and older, showing high mortality rates (15% of the total pig population), were selected for the study. Two groups were formed, each consisting of 4 deceased animals. The experimental group included pigs that died with signs of gastric ulcer disease, while the control group consisted of animals without any observed signs of stomach damage. Stomachs and local gastric lymph nodes were collected from pigs in both groups for further investigation.

Histological analysis. Stomachs and regional gastric lymph nodes from deceased piglets were fixed in a 10% formalin solution to perform histological analysis. Histological sections were prepared using a sledge microtome, followed by staining with hematoxylin and eosin. The histological specimens were examined using the hardware-software complex of the microscope "Leica DM 1000", digital camera "Leica DFC 295", and software "Leica Qwin 3.0".

PCR analysis. Nucleic acids (NA) extracts were subjected to polymerase chain reaction (PCR) to identify PCV2 genome presence in stomach tissue and lymph nodes of swine groups. Quantitative analysis of the genetic material of the pathogen in nucleic acid extracts was carried out using quantitative PCR with real-time detection of results using commercial tests EXOPOL (Spain). Amplification and detection of the results were carried out on a CFX 96 device (BioRad, USA). The thermal cycling protocol was applied in accordance with the instructions of the test systems used.

All samples were analyzed in technical triplicates, including appropriate negative controls (distilled water without DNA/RNA). PCR efficiency and correlation coefficients of standard curves ranged from 89.20% to 111.00%, and R2 values ranged from 0.988 to 0.999, indicating a fairly high level of linear dependence. The detection limit of qPCR was 10–100 plasmid copies from three independent assays. The DNA PCV2 content represented as gram equivalent (g.e.).

To test the linearity and dynamic range of qPCR, standard sample curves were generated by tenfold serial dilutions of plasmid DNA of known copy number. PCR results were calculated and analyzed using the CFX Manager program, and amplification efficiency (E) was estimated using the formula $E = (10^{-1}/\text{slope}) - 1$.

Western Blot. Relative content of interferon-α was estimated with western blot (WB) method as it described earlier (Kuryata et al., 2021) Briefly, the samples of protein were extracted from homogenates with Tris buffer pH 7.8 contained 0.2% SDS, 1.0% TritonX-100 and protease inhibitors cocktail during 60 min incubation at $+4^0$C. After incubation the homogenates were centrifuged at 20.000 g during 45 min. The supernatants were collected, mixed with Laemmli buffer and stored at -20^0C. Polyacrylamide gel electrophoresis (PAGE) with 4-20% gradient of acrylamide was used to separate the proteins. Separated proteins were transferred onto PVDF membrane with electric current intensity 250 mA for 120 min. After transferring the

membrane washed 3 times with PBS and incubated with primary anti interferon-α antibody (Abcam, ab191903) overnight at +4^0 C. Probed with primary antibodies membrane were washed with the same manner and probed to secondary HRP-conjugated anti-rabbit antibody (Abcam, ab205718) for 60 min at room temperature. After secondary incubation the membrane washed and developed with advanced ECL method with using green-sensitive X-ray film (Thermo Scientific, CL-XPosure Film).

Statistical analysis carried out with using "Statistics 6" software (StatSoft Inc, USA) where significant differences were accepted as $p < 0.05$.

Results

Pathological-anatomical observations were conducted to assess the frequency and area of ulcerative lesions on the surface of the stomach. The results of the analysis showed the presence of a thick layer of dense mucus on the mucous membrane, containing blood clots. The mucous membrane of the stomachs was soaked in blood and had a red color. Large rhomboid ulcers measuring 6 cm × 7 cm, 5 cm × 6 cm, 5 cm × 4 cm, and 4 cm × 2.5 cm were found, located in the cardiac region around the esophagus. The depth of the ulcers, on average, corresponded to 2/3 of the thickness of the muscular layer. The results of the pathological-anatomical studies are presented in Figures 1 and 2.

Intense reddening was observed in the fundal part of the stomach, along with superficial erosions and desquamation, as well as signs of chronic gastritis with glandular atrophy. The results of pathological-histological examination of stomach ulcer tissues showed a pathohistological picture of chronic, recurrent cardiac ulcers in pigs in the experimental group. Areas of necrosis with purulent-necrotic fibrinoid detritus, inflammatory infiltration, and granulation were evident at the edges of the ulcers (Fig. 3).

The formation of lymphatic follicles in the submucosal layer of the stomach mucosa may indicate the presence of pathogens of viral etiology. In the inflammatory infiltrate, a large number of fibroblasts and eosinophils were present in several cases (50%), which could be explained by the presence of mixed microflora (protozoa, fungi) (Fig. 4).

No signs of mucosal lesions in the cardiac part of pig

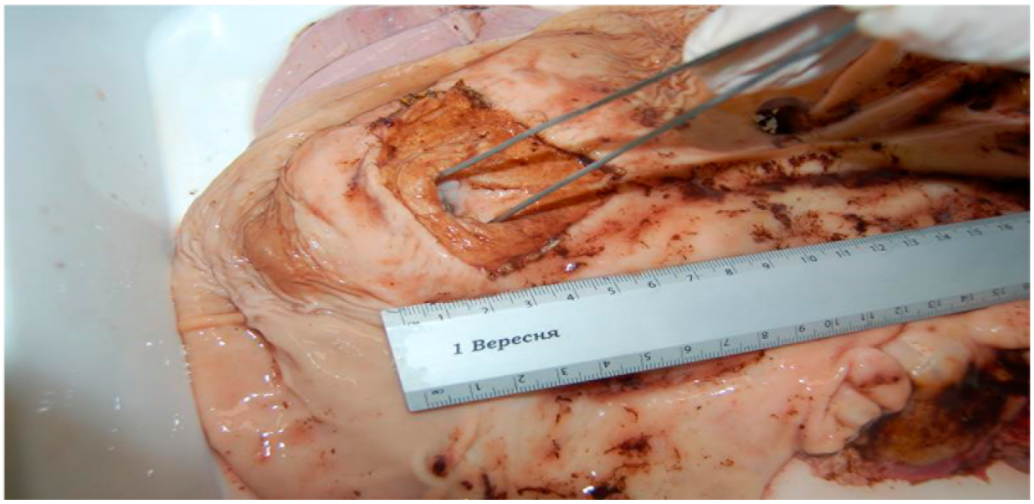

Fig. 1. The ulcer in cardiac area of the stomach.

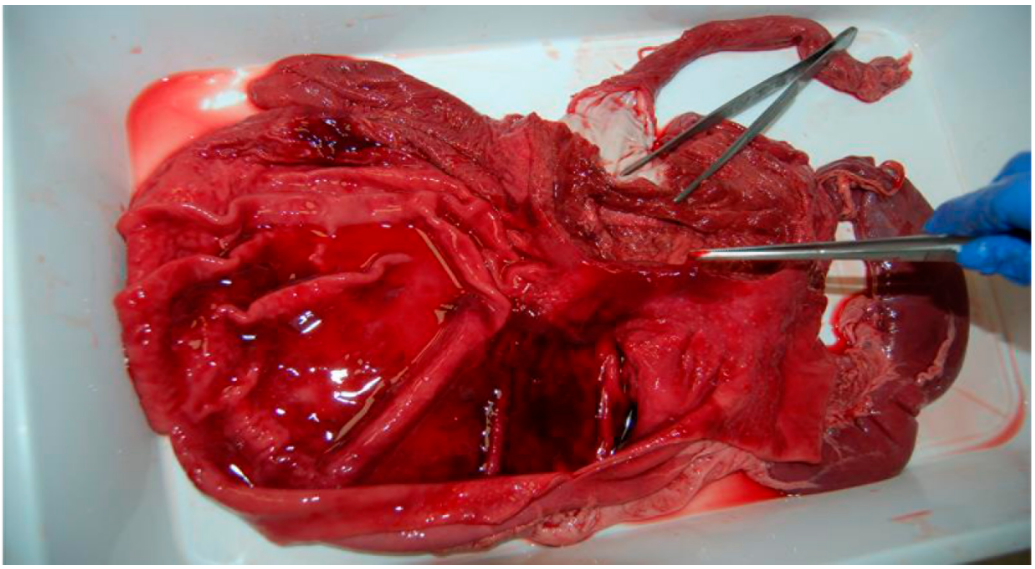

Fig. 2. Leakage of the mucous membrane of the sluts with blood.

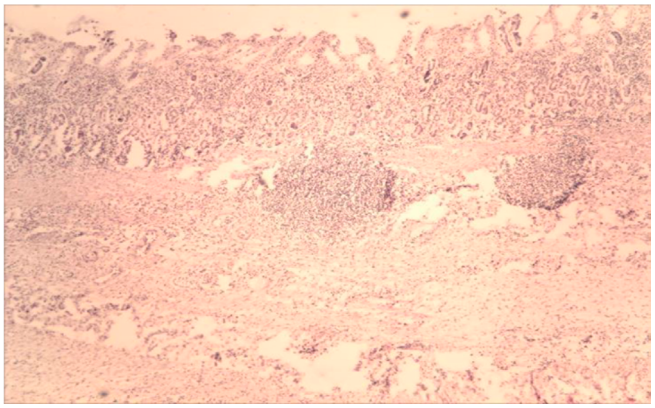

Fig. 3. Chronic atrophic gastritis at the acute stage with expression of erosions and formation of deep erosions (× 40)

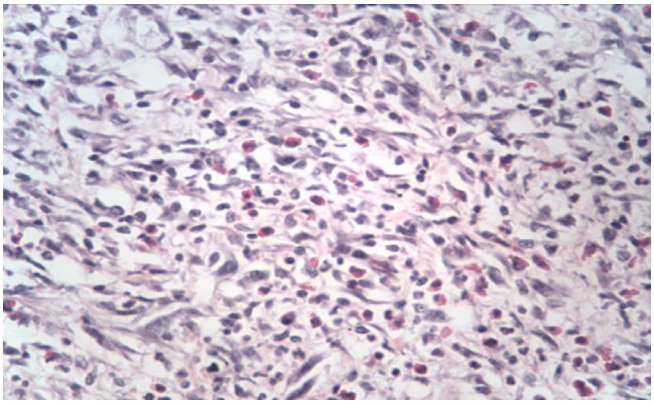

Fig. 4. Pro-inflammatory infiltrate into stomach tissue (× 400).

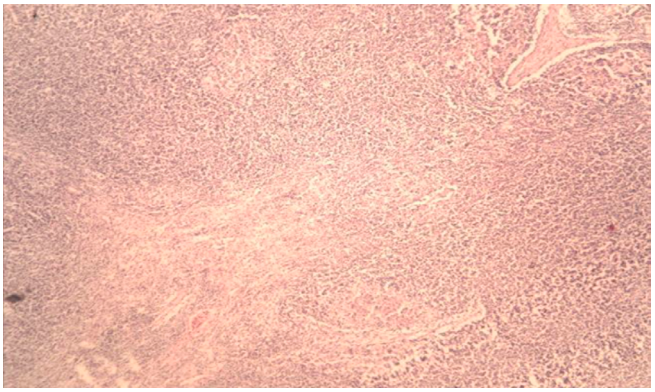

Fig. 5. Follicle structure disappearing (the index of immune system decline, × 40).

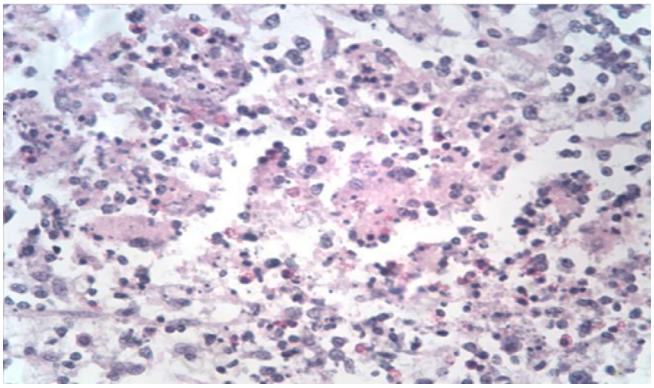

Fig. 6. Necrotic areas location (× 400)

stomachs were found according to the results of the pathological examinations of the animal stomach samples of the control group. The detected chronic mixed lymphadenitis in both gastric and mesenteric lymph nodes indicates the presence of inflammatory processes in the stomach. Furthermore, signs of the presence of protozoa (subcellular inclusions and eosinophils) were observed in the lymph nodes of animals from the experimental group (Fig. 5, 6).

Unlike the animals in the experimental group, the analysis of gastric lymph nodes in the control group pigs did not reveal any signs of inflammatory processes or pathogenetic changes. The lymph nodes exhibited clearly defined cortical and medullary portions, along with distinct sinuses, as confirmed by histological examinations.

The results of the PCR analysis did not detect the genetic material of PCV2 in the samples from the cardiac part of the stomachs of pigs in the control group. On the other hand, relatively high levels of PCV2 DNA were found in the ulcer tissues of animals in the experimental group (Table).

Obtained in present study results showed that the highest content of genetic material of PCV2 was found in the ulcer tissues of the cardiac part of the stomach, with a value of 5.63×10^{7}, compared to 2.02×10^{11} in the lymph nodes of pigs in the experimental group. At the same time, no DNA of PCV2 was detected in the samples taken from the pigs in the control group, neither in the stomachs nor in the lymph nodes. A significant parameter for the state of the body's antiviral defense system is the level of interferon production. The content of interferon-α was determined in the stomach samples of pigs to assess the production of this regulatory cytokine in response to circovirus infection and organic damage to the stomach tissue. The interferon-α content was measured in stomach tissue samples to assess the local production of this anti-viral cytokine. The relative content of interferon-αin the experimental group was nearly 18% lower compared to the control group 0.82 ± 0.112 and 1.00 ± 0.087, respectively (Fig. 7).

Overall, the results obtained in present study demonstrated an association between gastric ulcers in pigs and circovirus infection.

Table – The DNA PCV2 content in the gastric tissue and lymph nodes of the fattening swine (n = 4)

Animal groups	Cardiac gastric tissue	Lymph nodes
DNA PCV2 content in the experimental group, g.e., M ± m	$5.63\times10^{7} \pm 2.36\times10^{6}$	$2.02\times10^{11} \pm 0.92\times10^{11}$
DNA PCV2 content in the control group, g.e., M ± m	–	–
DNA PCV2 allocation in the experimental group, %	100	100
DNA PCV2 allocation in the control group, %	0	0

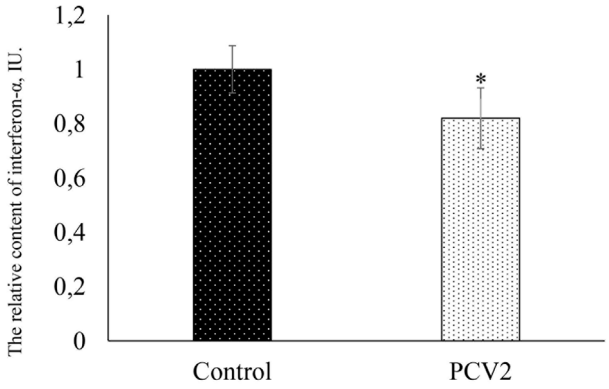

Fig. 7. Relative interferon-α content in stomach tissue of control and PCV2 groups. Significant differences in compare to control group: * – $p < 0,05$.

Discussion

Taking into the account high density and closeness of the animal location for industrial pig farming, circovirus infection has become a real threat to the economic efficiency of the industry. It is a highly contagious viral disease that leads to reduced growth rates in animals and the development of circovirus-associated syndromes, such as post-weaning multisystemic wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome (PDNS), which cause high mortality rates among piglets.

Infections with PCV2 can indeed suppress the host's immune system and enhance the infection and replication of other pathogens. However, the exact mechanisms through which this process occurs remain incompletely understood. The study of PCV2 viral infection revealed that persistent infection with the circovirus promotes bacterial agent modulation of macrophage polarization in vitro through targeting canonical signaling and epigenetic histones, thus contributing to bacterial co-infection and viral pathogenesis (Zhang et al., 2021).

It has also been found that PCV2 increases the risk of infection with other pathogens by inducing an immunosuppressive state in the swine organism (Choi et al., 2018, Noor et al., 2022). PCV2 is one of the major pathogenic factors causing dysfunction of endothelial cells, disrupting the cardiovascular system, initiating apoptosis processes in lymphocytes, and impairing cytokine secretion regardless of the swine's immune status. One of the current issues in modern industrial pig farming is the study of the main causes of gastric ulcers in pigs, the development of preventive measures, and thus the reduction of losses from culling and animal mortality.

Ulcerative lesions in pigs are more commonly located in the initial part of the non-glandular area of the stomach, near the entrance to the esophageal opening. PCV2 primarily enters through the mucous membranes. Epithelial cells play a crucial role in forming the barrier function of the intestinal system. The defense system of the gastric mucosa consists of a composite barrier comprising pre-epithelial, epithelial, and sub-epithelial elements. Mucus and superficial epithelial cells provide the next line of defense. The epithelial lining forms a regulated, selectively permeable barrier between the contents of the lumen and the underlying tissue layers. This permeability is partly determined by the most apical intercellular junction, known as "tight junctions."

While the pre-epithelial barrier is disrupted, the epithelial cells of the stomach bordering the site of injury can migrate to restore the damaged and this cell activity is a similar to other cell types (Nedzvetsky et al., 2021; Tykhomyrov et al., 2020). The pathogenesis and etiology of gastric ulcers in pigs, as well as the mechanisms of action of causative factors, remain unclear. A particular issue is the search for molecular markers that reflect the contribution of individual causative factors to the development of ulcers.

The obtained results of our study have demonstrated the usefulness and potential of evaluating the epithelial barrier of the digestive system in pigs to identify the causative factors involved in ulcer formation. In recent years, significant progress has been made in understanding the molecular mechanisms of the antiviral action of interferons, as well as the mechanisms by which viruses counteract interferon-mediated host defense. Numerous studies have revealed the complexity of the interferon system, which functions as an integral part of the immune system in the body (Chen et al., 2016, Mutthi et al., 2018, Kang et al.,2021, Wang et al., 2022).

The results of present study showed that the production of interferon-α was lower in tissue samples affected with ulcers compared to the stomach tissues of the control group of pigs. This decline may be associated with the immunosuppressive properties of circovirus, which suppress cytokine production. Considering that interferon-α is a regulatory cytokine synthesized by most cell types in response to various cytotoxic factors, including viral RNA and DNA, insufficient interferon production in affected areas promotes the localization of circovirus within them.

The results obtained in the study regarding the decrease in interferon in ulcerated areas indicate possible disruptions in the production of this cytokine. These disruptions are caused by the proliferation of circovirus and suppression of the immune response specifically in the damaged areas of the stomach. Additionally, the observed decrease in interferon production in the damaged areas of the stomach suggests the involvement of innate immunity in the pathogenesis of gastric ulcer disease in pigs during the fattening period. Interferon-α is considered an anti-inflammatory cytokine involved in regulating the balance of the response to the initiation of pro-inflammatory reactions (Gu et al., 2012, Razzuoli et al., 2013, Chen et al., 2016).

Thus, the generation of gastric ulcers in pigs may be associated with circovirus-induced cellular damage and disruption of interferon-α production. This may lead to an imbalance in the expression of pro-inflammatory and anti-inflammatory factors, resulting in a chronic inflammatory process and local cytotoxic effects in ulcerated areas.

Conclusions

The present results demonstrated decline in interferon-α production, mucosal immune suppression and decreased immune defense of the gastrointestinal system in animals against the background of PCV2 proliferation in local ulcerated areas. In the

ulcer tissues, a decrease in the content of interferon-α by 18% ($p < 0.05$) was observed compared to the values in the undamaged stomach tissue of animals in the control group.

All detected ulcers were located in the cardiac part of the pig's stomach and had an average area of 25.5 ± 6.85 cm^2.

PCV2 DNA was found in the stomach tissues and lymph nodes of pigs afflicted with ulcerative disease, with quantities measuring $5.63\times10^7 \pm 2.36\times10^6$ g.e. and $2.02\times10^{11} \pm 0.92\times10^{11}$ g.e., respectively. Conversely, PCV2 DNA was not detected in pigs lacking indications of ulcerative disease.

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