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ИЗУЧЕНИЕ ЭКСПРЕССИИ ФУНКЦИОНАЛЬНО-ЗНАЧИМЫХ ГЕНОВ ПРИ ТЕРАПИИ КОРОНАВИРУСНОЙ ИНФЕКЦИИ У ЦЫПЛЯТ

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Изучен уровень экспрессии противовоспалительных цитокинов NF-kB, IL-6, INF-γ, Caspasa-3, Fc у кур в легких и кишечнике при моделировании инфекционного бронхита. Для моделирования коронавирусной пневмонии вакцину вводили индивидуально, 10 доз на голову перорально. Цыплятам 1-й опытной группы скармливали препарат Люманце в расчете 3 кг/т корма, 2-й опытной – препарат Глицевир в расчете 200 мкг/0,3 мл на голову. Цыплята контрольной группы препараты не получали. Выявлено, что противовирусные препараты в опытных группах подавляли разрушение эпителиальных клеток в кишечнике. Это не всегда может свидетельствовать о позитивном характере, поскольку в случае апоптоза происходит разрушение не только пораженных вирусными частицами клеток кишечника, но и здоровых. Отмечено снижение количества активных макрофагов в кишечнике опытных групп относительно контрольной. Количество вырабатываемого интерферона также находилось ниже контроля, что свидетельствует о пониженной активности иммунной системы. Выявлена более высокая провоспалительная активность в респираторной системе цыплят при использовании Глицевира. Она заключается в повышенном уровне экспрессии генов IL-6, интерферона-гамма, рецептора макрофагов к Fc фрагментам антител, фактора регуляции воспаления NF-kB в сравнении с препаратом Люманце, обладающим противовоспалительной активностью, но и в сравнении с цыплятами контрольной группы, не подвергавшихся лечению. Сделан вывод о возможности прогнозирования риска развития обострения инфекционного процесса в легких на фоне локального снижения вирусной нагрузки в кишечнике. Необходим комплексный подход при терапии коронавирусных инфекций, включающий или противовирусные препараты системного действия, или противовоспалительные средства.

Ключевые слова: ген, интерлейкин, коронавирус, Люманце, Глицевир, цыплята

STUDY OF THE EXPRESSION OF FUNCTIONALLY RELEVANT GENES IN THE TREATMENT OF CORONAVIRUS INFECTION IN CHICKENS

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The level of expression of anti-inflammatory cytokines NF-kB, IL-6, IFN-y, Caspasa-3, FC in chickens in the lungs and intestines during the modeling of infectious bronchitis in chickens was

studied. To simulate coronavirus pneumonia, the vaccine was administered individually, 10 doses per head orally. The chickens of the 1st experimental group were fed with the Lyumantse preparation at the rate of 3 kg / t of feed, the 2nd experimental group received the Glitsevir drug at the rate of 200 μg / 0.3 ml per head. The chickens of the control group did not receive the preparations. It was revealed that antiviral drugs in the experimental groups suppressed the destruction of epithelial cells in the intestine. This may not always be an indication of a positive character, as in the case of apoptosis, not only the intestinal cells affected by the virus particles but also healthy cells are destroyed. There was a decrease in the number of active macrophages in the intestines of the experimental groups relative to the control. The amount of interferon produced was also below the control, which indicates a decreased activity of the immune system. A higher pro-inflammatory activity in the respiratory system of chickens was detected when Glicevir was used. It consists of increased expression of IL-6, interferon-gamma, macrophage receptor to Fc antibody fragments and inflammatory regulatory factor NF-kB genes compared to Lumantse with anti-inflammatory activity, but also compared to untreated control group chickens. It is concluded that it is possible to predict the risk of an exacerbation of an infectious process in the lungs against the background of a local decrease in the viral load in the intestine. An integrated approach is needed in the treatment of coronavirus infections, including either systemic antiviral drugs or anti-inflammatory drugs.

Key words: gene, interleukin, coronavirus, Lyumantse, Glitsevir, chickens

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INTRODUCTION

In modern science, pro-inflammatory and anti-inflammatory cytokines are considered as the most important factor of intercellular and systemic interaction in the body. Cytokines are a group of endogenous glycosylated polypeptide mediators of intercellular interaction involved in the formation and development of the body's defense reactions when pathogens are introduced and tissue integrity is compromised, as well as in the regulation of a number of normal physiological functions [1].

The state of the body's immune functions is largely determined by the ratio of pro- and anti-inflammatory cytokines. Functional antagonism between them has been shown to exist (2). An acute increase in circulating cytokine levels leads to the development of a protective systemic inflammatory response, often referred to as a cytokine storm. Pathologically high concentrations of pro-inflammatory cytokines can cause septic shock and death.

In situations in which cytokine levels exceed the physiological concentration for a prolonged period, they no longer mediate protection, but

become mediators of pathology [3-6]. This is also population-driven: the organism must be eliminated to prevent further spread of the pathology.

Interleukin 6 (IL-6) is an immunoregulatory cytokine with a broad spectrum of action: it regulates the processes of inflammation and cell division. IL-6 is synthesized from macrophages, T cells, fibroblasts, vascular endothelial cells, glial cells and epithelial cells after interaction with pathogenic molecules. In the formation of the immune response, interleukin 6 is involved in the production of antibodies. An excess of this cytokine leads to the development of an autoimmune reaction and tissue damage (7).

Caspases are a family of cysteine proteases that are involved in the cleavage of peptide bonds. The expression of caspases-3, -8, -9 is an indicator of the cytotoxicity of the apoptotic stimulus, making these markers an important part of research into the processes of apoptosis in the body [8].

Cells of the immune system interact with each other using cytokines - modulators of immune responses. Among these, interferons occupy an important place. They have antiviral and antibacterial effects and are involved in the anti-tumor immune response [9]. INF-γ, like most cytokines, has pleiotropic effects and plays an important role in the immune response. Initially, INF-γ was thought to be produced only by natural killer (NK) cells, CD4+ Th1-lymphocytes and cytotoxic CD+ T-lymphocytes. Later, it became known that B-lymphocytes, NKT cells and antigen-presenting cells (APCs) (macrophages, dendritic cells) are also able to secrete this cytokine [10].

NF-kB is found in almost all animal cell types and is involved in cellular responses to stimuli: stress, cytokines, free radicals, heavy metals, ultraviolet irradiation, oxidized LDL, and bacterial or viral antigens. NF-kB plays a key role in regulating the immune response to infection (11,12).

The antiviral effect of medicines is often detected by qRT-PCR [13–15], IFA [16, 17].

Several reports indicate that MDA5 signaling pathways and cytokines of innate immunity are activated following infection with IBV strain M41 [18]. The MDA5 signaling pathway is disrupted by cleavage of the adaptor protein MAVS in the IBV JS/2010/12 infection strain [19]. The INF type I response plays a crucial role in resistance to the IBV strain SAIBK2 [20]. The MDA5 signaling pathway and innate immunity cytokine (NF-kB and IRF3) were induced following infection with IBV-M41 strain [17]. Researchers found that mRNA expression levels of MDA5, MAVS, INF-α, INF-β, NF-kB, TNF-α and IL-6 were significantly increased following infection with IBV in vitro and in vivo [18].

The purpose of the study was to evaluate the expression of IL-6, Fc, NF-kB, Caspasa-3 and INF-γ genes during coronavirus infection in chickens.

MATERIAL AND METHODS

Chickens of the Shaver cross were vaccinated against infectious bronchitis in chickens being 14 days of age (IBC vaccine, strain Ma5, live dry). To simulate coronavirus pneumonia, the vaccine was administered individually, 10 doses per head orally. Chickens of experimental group 1 (n = 10) were fed Luymantse at the rate of 3 kg/t feed, and those of experimental group 2 (n = 10) - Glitsevir (glycyrrhizic acid derivative in chitosan nanoparticles) at the rate of 200 μg/0.3 ml per head. The concentrate was diluted by a factor of 3. Chickens of the control group (n = 11) did not receive the drugs.

On the 22nd day, the birds were slaughtered by cervical-cerebral dislocation and autopsied. RNA was isolated from internal organs by phenol-chloroform extraction, and RT-PCR was performed. The cDNA synthesis was performed using oligonucleotide N7.

To confirm the success of infection, the presence of IBK virus genomic RNA was tested by real-time RT-PCR, PCR was performed in a final volume of 20 μl containing 67 mM Tris.-HCl (pH 8.9), 16 mM (NH4) 2SO4, 2.4 mM MgCl2, 0.01% Tween 20, 0.2 mM dNTP, 0, 3 mM oligonucleotide primer solutions 5`-atgctcaaccttgtccctagca-3`5`tcaaactgcggatcacgt-3` and FAM probe tggaagtaggaccaac-BHQ, 1-2 units HotStart Taq-DNA polymerase. PCR was

performed on a CFX amplifier (BioRad) according to the following program: initial denaturation - 95 °C (15 min), then 40 cycles: denaturation - 95 °C (10 s), annealing - 60 °C (30 s).

RNA extractability from samples was monitored using real-time PCR specific to the housekeeping gene glyceraldehyde3-phosphate dehydrogenase (GAPDH) mRNA using the protocol above, using primers 5`cgtgaccccagcaacatcaa3` and 5` acttaccccagccttccat3` taqman ROX probe tggagtactggtcttccacc- BHQ2.

The expression of IL-6, Fc, NF-kB, Caspasa-3, INF-γ genes was assessed by the delta-delta Ct method relative to the expression level of housekeeping genes (see the table).

RESULTS AND DISCUSSION

The preparations Lyumantse and Glitsevir inhibited the destruction of epithelial cells in the intestines of the experimental groups (see Figure 1). This is not necessarily indicative of a positive character, since in the case of apoptosis, not only healthy but also infected cells are destroyed. A decrease in the number of active macrophages in the intestine relative to the control group was observed. The amount of interferon produced was also lower than the control, indicating a reduced activity of the immune system.

Glitsevir consists of nanoparticles, which are not absorbed by the intestine and have a local effect. Lyumantse can be absorbed as it contains salts of butyric acid and has a systemic effect in addition to its local effects. In the intestine, both preparations reduced the viral load.

Glitsevir provoked more intensive expression of genes involved in the immune response and inflammation, characterized by increased levels of NF-kB, IL-6, INF-γ (more T helper cells encountering viral particles), Fc (more macrophages) and Caspasa-3 (cells in the lungs are destroyed more intensively) (see figure 2).

The suppression of epithelial cell destruction by the experimental preparations is also evidenced by the reduced specific fraction of the Fc-fragment relative to the control group. Fc-receptor is present on macrophages and is responsible for antibody binding to macrophage. A decrease in the number of active macrophages in the intestine relative to the control group can be asserted. Caspases play an important role in the development and regulation of apoptosis and inflammation. In this case (as the coronavirus itself has no effect on cell destruction) there is destruction of the affected cells by immune agents such as T-killers.

Interferon gamma (INF-γ), which is a pro-inflammatory cytokine and activates many cells (T cells, B cells, etc.), is produced when T-helper cells interact with antigen. In the intestine the amount of interferon produced is reduced, this also indicates a reduced activity of the immune system.

Interleukin-6 is a pro-inflammatory cytokine, the amount of which directly affects the development of a cytokine storm. IL-6 creates

Нуклеотидные последовательности праймеров ПЦР для оценки экспрессии генов
PCR primer nucleotide sequences for assessing gene expression

Interleukin	Primer	Source reference	PCR program			
IL-6	5`-aaatccctcctcgccaatct-3` 5`-ccctcacggtcttctccataaa-3	[21]	1	95 °C	7 min	1 cycle
Caspasa-3	5`-ccaccgagataccggactgt-3` 5`-aactgcttcgcttgctgtga-3`	[22]				
INF-γ	5`-cactgacaagtcaaagccgc-3` 5`-accttcttcacgccatcagg-3`	[22]	2	95 °C	15 s	39 cycles
FcR	5`-cactgacaagtcaaagccgc-3` 5`-accttcttcacgccatcagg-3`	[22]				
NF-kB	5`-tcaacgcaggacctaaagacat-3` 5`-gcagatagccaagttcaggatg-3`	[22]	3	64,5 °C	30 s	
			END			

The data were processed by methods of variation and nonparametric statistics.

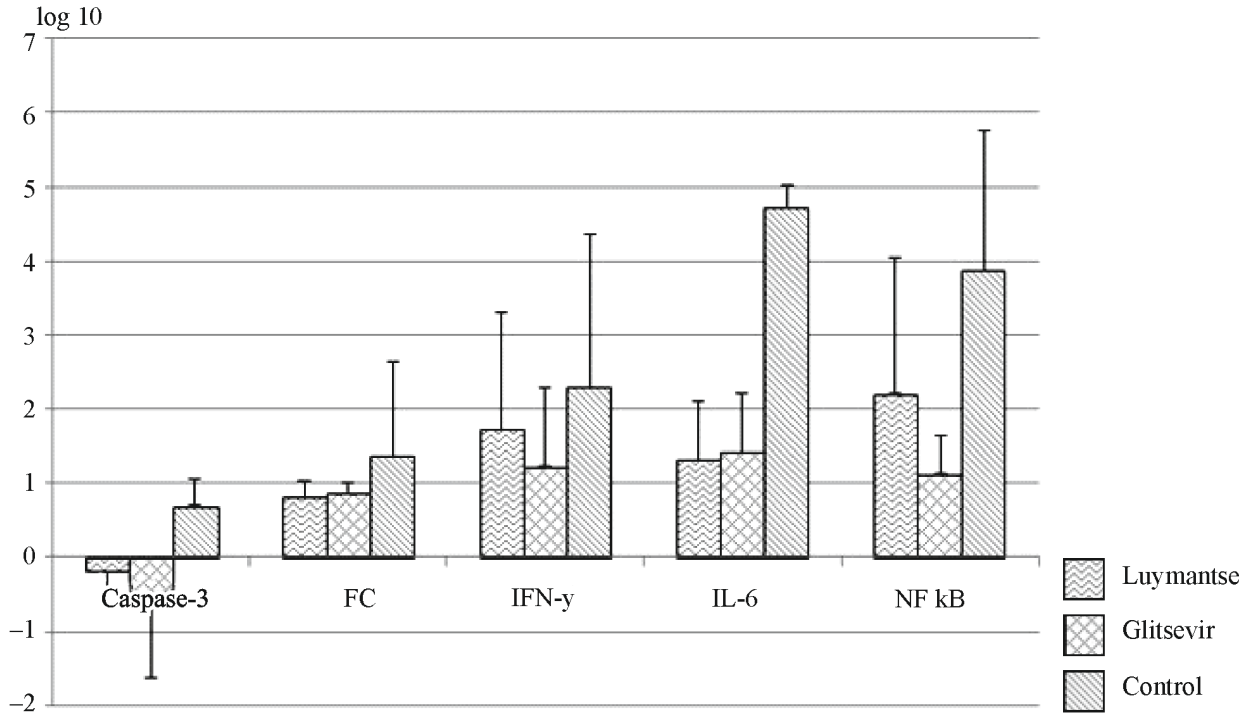

Рис. 1. Экспрессия генов в кишечнике у птицы опытной и контрольной групп, log10(ddCt) (относительно GAPDH)

Fig. 1. Gene expression in the intestine of birds in the experimental and control groups, log10(ddCt) (relative to GAPDH)

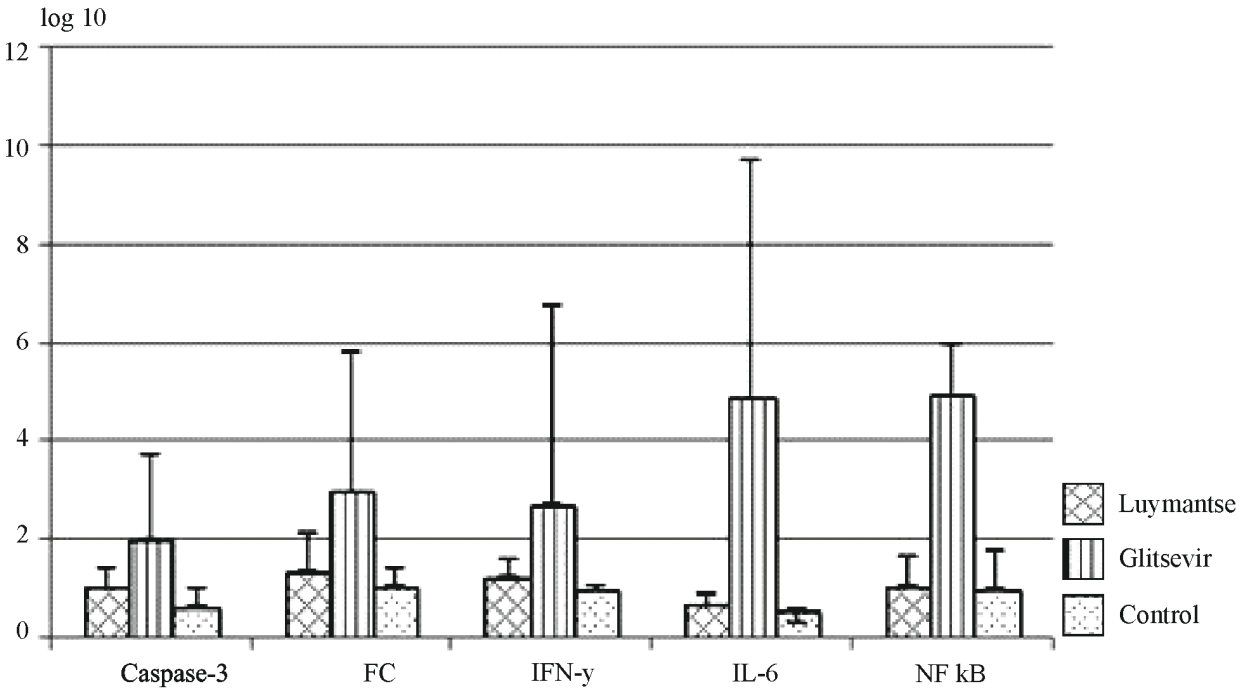

Рис. 2. Экспрессия генов в легких у птицы опытной и контрольной групп, log10(ddCt) (относительно GAPDH)

Fig. 2. Gene expression in lungs of poultry from experimental and control groups, log10(ddCt) (relative to GAPDH)

conditions in the body that overload the immune system and two pathways emerge: elimination of the pathogen in its entirety or elimination of the infected animal itself (7, 11). At the moment when viral antigens meet toll-like receptors, NF-kB is activated, which in turn is the trigger mechanism for both the innate immune response (macrophage migration, etc.) and adaptive immunity (antibody production, T-cell formation). NF-kB is the earliest factor and is most actively exploited in coronavirus infections. It is manifested by abnormally prolonged migration of neutrophils, granulocytes to the focus of inflammation, their activation, and increased proliferation, whereas in a normal infection there is only a short period of neutrophils fighting the primary focus of infection. This mechanism can subsequently lead to a leukotriene storm, subsequently to a cytokine one.

Glycyrrhizic acid has antiviral, anti-inflammatory, antipruritic and immunomodulatory effects, affects different types of virus DNA and RNA in vitro and in vivo, interrupts virus replication at early stages, causes virion exit from the capsid, preventing its penetration into cells, which is due to selective dose-dependent inhibition of phosphorylated kinase. Glycyrrhizic acid interacts with virus structures, changing different phases of the viral cycle, which is accompanied by irreversible inactivation of viral particles that are free outside cells, blocks the introduction of active viral particles inside cells, disrupts the virus ability to induce synthesis of new viral particles, induces interferon formation, which is a component of antiviral action, inactivates these viruses in nontoxic concentrations for normally functioning cells . The anti-inflammatory activity of glycyrrhizic acid is combined with a stimulating effect on humoral and cellular immunity factors[1].

The difference between the activity of drugs with a resorptive effect (Lyumantse) and those without (Glitsevir in chitosan nanoparticles) is the higher pro-inflammatory activity in the respiratory system with Glitsevir. It consists in increased expression of IL-6, interferon-gamma, macrophage receptor to Fc antibody fragments, inflammatory regulation factor NF-kB genes not only in comparison with Lyumantse, which possesses anti-inflammatory activity due to oily acid, but also in comparison with an untreated control group of chickens.

We propose two hypotheses about the effect of the drugs on the affected intestine:

– both drugs reduce inflammation in the intestine by suppressing immunoreactivity and therefore reducing the immune response;

– the drugs reduce the viral load, so there is less presentation of viral antigens and less production of immunocompetent cells.

In either hypothesis, Glitsevir had a suppressive effect on the inflammatory processes in the intestine without affecting the lungs (see Figure 1).

The main theory is a decrease in the activity of immunocompetent cells within the gastrointestinal tract, primarily Treg lymphocytes (active producers of the anti-inflammatory cytokine IL10) due to the suppression of the infection process locally, by the site of action of Glitsevir, i.e. in the intestine.

This assumption is supported by the suppression of IL-6 gene expression activity, a direct antagonist of the pro-inflammatory cytokine IL10 and cells producing this interleukin. All blood and lymph from the gastrointestinal tract necessarily pass through the lungs, i.e. virtually undiluted, so the contribution of the regulatory activity of the intestine immune system towards immunocompetent cells in the lungs must be substantial. Thus, the risk of an exacerbation of the infectious process in the lungs against the background of a local reduction of the viral load in the intestine can be predicted, which requires a comprehensive approach in the therapy of coronavirus infections, including either antiviral drugs of systemic action or anti-inflammatory drugs.

[1] Patent RF № 2044145 (Russian Federation). Di- and trinicotinates of glycyrrhizic acid and inhibitor of human immunodeficiency virus reproduction / G.A. Tolstikova, L.A. Baltina, K.P. Volcho, O.A. Plyasunova, A.G. Pokrovskii, N.F. Salakhutdinov. Published 10.08.2007. Bulletin number 22.

CONCLUSIONS

1. A statistically significant suppression of pro-inflammatory cytokine IL-6 expression ($p < 0.05$) in experimental groups of chickens treated with Lyumantse and Glitsevir was detected in a model of coronavirus infection.

2. The application of Glitsevir in chitosan nanoparticles was limited to local action, which was characterized by absence of suppression of proinflammatory cytokines in lungs and, on the contrary, differed from the Lyumantse action by increased dispersion of expression levels of the studied genes and average levels of increase in expression of IL-6, NF-kB, INF-γ (proinflammatory cytokines) and Caspasa-3, Fc-fragments in lung tissue.

3. To reduce the risks of acute pneumonia in coronavirus infections, the use of topical antiviral drugs in the intestine should involve the use of systemic anti-inflammatory drugs.

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