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Effectiveness of Combined Use: Inactivated Vaccines with Immunostimulants on the *In vivo* Model of Teschen Disease

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Abstract | The Teschen disease (enzootic encephalomyelitis, enterovirus encephalomyelitis in pigs) is a viral disease in pigs, characterized by the development of non-suppurative encephalomyelitis and the emergence of paralysis. Live and inactivated vaccines are used to prevent the Teschen disease. The vaccines are prepared from tissues of the central nervous system of infected pigs or using a cultured viral suspension with the addition of various adjuvants. The inactivated vaccine against the Teschen disease protects 70-80% of vaccinated pigs from subsequent intracerebral infection with a pathogenic virus. The titer of virus-neutralizing antibodies reaches a maximum (1:64 - 1:1444) two weeks after the second vaccination. Vaccinated pigs with titer of neutralizing antibodies above than 1:32-1:128 are resistant to infection enterovirus encephalomyelitis of pigs. If the animal was older than 6 weeks age at the time of vaccination expressed antibody formation was observed. The disadvantages of inactivated vaccines are the low level and short duration of the formed protective immunity. Therefore, to induce and maintain a high level of humoral immunity people are forced to resort to multiple vaccinations. Another way to amplify the protectiveness of inactivated vaccines is the use of immunomodulators for the regulation of immunological reactivity. A wide field of activity remains for the selection of effective immunomodulators and optimal schemes for their combined use with inactivated vaccines. It seems advisable to determine the possibilities of using a number of immunostimulants produced by the domestic bioproductivity in the vaccine prophylaxis of viral diseases of farm.

Keywords | Teschen disease, Viral disease, Domestic pigs, Immunostimulants, Vaccine prophylaxis, Tissue culture infective dose.

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INTRODUCTION

The Teschen disease (enzootic encephalomyelitis, enterovirus encephalomyelitis in pigs) is a viral disease in pigs, characterized by the development of non-suppurative encephalomyelitis and the emergence of paralysis. All age groups of domestic pigs are sensitive to the disease, but in the greater risk zone is the young of pigs (Barinsky et al., 2015; Romantsov et al., 2004). Teschovirus is a genus of viruses in the order Picornavirales, in the family Picornaviridae. Pigs serve as natural hosts (Barinsky et al., 2012).

The incubation period lasts 1-4 weeks. In the prodromal period, rarely exceeding 72 hours, low temperature, loss of appetite, sometimes vomiting, acute rhinitis, movement coordination disorder are noted. Often these violations go unnoticed. Then there are clinical signs of Central nervous system (CNS) lesions: twitching of various muscle groups, involuntary movement, weakness muscles of the limbs, nystagmus, etc. (Binhong et al., 2019; Chernigova et al., 2019). Then symptoms destruction spinal brain develop - staggering tense gait, weakness limbs, incomplete their leaning on land, progressive paralysis, gradually seizing

limbs, a neck and head. Due to paralysis of the pharynx, partial or complete loss of voice is noted. In severe cases, the disease usually ends in coma and death of animals. With a lighter flow, a difficult gait is noted, the development of semi-paralysis and paralysis without the phenomena of excitation. Animals are usually infected alimentary (Kulikov et al., 2016; Kulikov et al., 2015). Then the virus is reproduced in the mucous membrane epiteliocytes of the large intestine, and from there it penetrates into the lymph nodes, bloodstream and spreads throughout the body, including the CNS. Clinically, the disease proceeds with the phenomena of acute encephalomyelitis and meningitis, the most pronounced depression, increased skin sensitivity, spastic state of the labial, ocular and masticatory muscles, as well as the muscles of the limbs, accompanied by swimming movements; vomiting, aphonia, hoarseness, strabismus, grinding teeth, difficult hard breathing are often observed. After the onset of the stage of paralysis, the body temperature decreases to normal and before death reaches 32-34 °C. Significant pathological changes are found only in the CNS. They are characterized by hyperemia of the meninges and serous infiltration. On sections of the brain in various places, hemorrhages, perivascular round-cell infiltration with the formation of foci of neuronophagy are noted, and only the gray matter of the brain is affected. In the cerebellum, changes are found in Purkinje cells. The most characteristic changes are found in ganglion cells. Affects the motor centers of the ventral horns (liquefaction of the cell nuclei, the formation of vacuoles, the presence of karyorhexis). Significant prevalent neuronophagia in the ventral horns of the lumbar region of the spinal cord is noted. Diagnostic value is represented by histological changes. They are most pronounced in the middle and caudal parts of the brain (especially at its base-the legs of the brain), as well as in the cervical and lumbar parts of the spinal cord (Seleznev et al., 2017).

At pathoanatomic research observe a hyperemia of soft covers, the phenomena of edema of gray matter (mainly rhomboid and middle brain), cervical and lumbar thickenings of a spinal cord, catarrhal rhinitis, bronchitis and pulmonary edema, dot and spotty hemorrhages under EPI- and endocardium. The stomach is usually filled with fodder masses, the mucous membranes of it and the large intestine are folded, focal hyperemic and covered with a thick transparent mucus. The bladder is always full (Sachivkina et al., 2009; Stanishevskiy et al., 2017).

Microscopic examination of the Central nervous system always determine non-purulent, lymphocytic type of polyencephalomyelitis and meningitis. In the study of animals that fell in the first days of the Teschen disease, in the gray matter of the brain and spinal cord, different focal and diffuse cell infiltrates from glial elements, histiocytes and lymphocytes are observed. Among the cells of the infiltrate, small clusters of red blood cells are sometimes found. Polymorphonuclear and neutrophilic leukocytes are rare and only in animals that have fallen (or killed) in the first hours and days of clinical manifestation of the disease. Many infiltrate cells are in a state of pycnosis and rexis. Disintegration of nervous tissue, dystrophic and necrobiotic changes of nerve elements, decay and disappearance of phospholipids are often registered in lesions (Vatnikov et al., 2019).

Live and inactivated vaccines are used to prevent the Teschen disease. The vaccines are prepared from tissues of the central nervous system of infected pigs or using a cultured viral suspension with the addition of various adjuvants. The inactivated vaccine against the Teschen disease protects 70-80% of vaccinated pigs from subsequent intracerebral infection with a pathogenic virus. The titer of virus-neutralizing antibodies reaches a maximum (1:64 - 1:1444) two weeks after the second vaccination. Vaccinated pigs with titer of neutralizing antibodies above than 1:32-1:128 are resistant to infection. If the animal was older than 6 weeks age at the time of vaccination expressed antibody formation was observed. The disadvantages of inactivated vaccines are the low level and short duration of the formed protective immunity. Therefore, to induce and maintain a high level of humoral immunity people are forced to resort to multiple vaccinations. Another way to amplify the protectiveness of inactivated vaccines is the use of immunomodulators for the regulation of immunological reactivity (Donchenko et al., 1989; Dudnikov et al., 1995; Ponomarev et al., 1999) The results of experimental studies using the example of mice with tick-borne encephalitis showed reliable protection in experimental prophylaxis with ridoctin and polyribonate in combination with a specific inactivated vaccine. In addition, a similar vaccination scheme is recommended for emergency prophylaxis of tick-borne encephalitis in the infection centers focuses on infection (Buzun et al., 1998; Marakhova et al., 2016).

The use of the Polyoxidonium-Vet Solution immunomodulator allows to increase titers of specific antibodies against parainfluenza-3 while vaccinating calves with the inactivated vaccine "Combovac-R" on average three times in comparison with the control group vaccinated without using the immunomodulator (Romantsov et al., 2004; Stanishevskiy et al., 2017). At the same time, it should be noted that hitherto immunostimulants are practically not used in the vaccine prophylaxis of animal infectious diseases. Therefore, a wide field of activity remains for the selection of effective immunomodulators and optimal schemes for their combined use with inactivated vaccines (Sachivkina et al., 2009; Sereda et al., 2001). It seems advisable to determine the possibilities of using a number of immunostimulants produced by the domestic bioproductivity in the vaccine prophylaxis of viral diseases of farm.

MATERIAL AND METHODS

Animals: outbred guinea pigs with a body weight of 300 - 400 g, gilts of large white breed with a body weight of 30-40 kg, 3.0-3.5 month old rabbits with a body weight of 1.5-2.5 kg. Vaccines: "The cultural emulsified vaccine against Teschen's disease", "The inactivated cultural vaccine against infectious rhinotracheitis" produced by the Federal State Budget Scientific Institution "Federal Research Center for Virology and Microbiology". Commercial immunostimulants: N-acetyl-glucosaminyl-N-acetylmuramyl-L-alanine-D-isoglutamine (GMDP, Glycopid), Fosprenyl (disodium salt of polyprenol phosphate, 4 mg/cm^3), Prestimol (polysaccharide derived from the culture of the fungus Saccharomycode ludwigit), Immunophan (synthetic hexapeptide: arginil-alpha-aspartyl-lysil-valil-tyrosil-arginine, 50 mcg/cm^3), Ribotan (a mixture of low-molecular polypeptides, not less than 10 mcg/cm^3, and fragments of yeast RNA, not less than 10^5 mcg/cm^3) (Ivanova-Radkevich et al., 2019; Stanishevskiy et al., 2017; Staroselov et al., 2018). Teschen disease virus, "Navlya" strain, with a titer of 5.5-8.0 lg TCID50 (tissue culture infective dose)/cm^3; infectious rhinotracheitis virus (IRT) of cattle, "TK–A" strain, with a titer of 7.0 lg TCID50/cm^3 were used from the State collection of microorganisms of Federal Research Center of Virology and Microbiology.

The growth medium was removed and the virus was introduced on a supporting medium with a multiplicity of infection 0.01 TCID50/cell to obtain infectious material of Teschen disease viruses or IRT of cattle from mattresses with 2-day transplanted culture of pig testicles cells. The infected cell culture was incubated in stationary conditions at 37 °C for 2-3 days till the development of TCID. Then the virus-containing material was frozen, thawed and stored at minus 70 °C.

The test serum of the blood of animals sequentially diluted twice with medium MEM (Minimum Essential Medium) for the detection of neutralizing antibodies. Then equal volumes of 0.2 cm^3 serum and Teschen disease virus, or IRT of cattle, were mixed in cells of a 96-well microliter plate and incubated for 2 hours at 37 °C. After this period, 0.1 cm^3 mixture of virus and serum was introduced into the sensitive cell culture. At the same time, the cells and the virus were monitored. After 72 - 96 hours, the reaction was recorded. As the serum titer was taken the highest serum dilution, ensuring the preservation of the cell monolayer in 50% of the wells.

Heparin-stabilized blood was used at a rate of 25 UA (unit of activity) per 1 cm^3 of blood to isolate lymphocytes. Blood was diluted 3 times in volume by culture medium. 10 cm^3 of the ficoll-pack solution was poured into sterile test-tube with a volume of 20 cm^3. The cell mixture was carefully layered over the wall of the tube. The ratio of the cell mixture to the separating solution was 2:1-4:1. The test-tubes were capped and centrifuged for 30 min at 8 °C and 700 g. (Khan et al., 2018; Sachivkina et al., 2009) Washing of the lymphocytes was carried out by centrifugation at 500 g for 20-30 minutes at least 2 times. Each time, a suspension of lymphocytes was diluted 2-5 times by volume with RPMI-1640 medium (Roswell Park Memorial Institute medium). The precipitate was resuspended to the cell concentration was no fewer than 4 million/cm^3. 0.1 cm^3 of a suspension of lymphocytes and 0.1 cm^3 of a working dilution of mitogen were imported into experimental alveolus. 0.1 cm^3 of a suspension of lymphocytes and 0.1 cm3 of culture medium mitogen were imported into control alveolus. Microplates were placed in a CO_2 incubator (37 ° C, 95% humidity, 3% CO_2) for 72 hours. 0.025 cm^3 of a 3H-thymidine solution with an activity of 4 MBq/cm^3 was added to each well by 16-18 hours before the end of the incubation. The cells were transferred to glass-fiber filters by a cell collector, washed 8 times with distilled water 0.1 cm^3 per alveolus. The cell filters were dried for 3-4 hours at 37 °C and transferred to vials containing 3 cm^3 of ZhS-107, after which the radioactivity was determined on a scintillation counter. The counting results were presented as a stimulation index (7, 8). The significance of differences was assessed by Student t-test.

Ethical Approval

All the experiments were conducted in accordance with the legislation (European Convention for the Protection of Vertebrate Animals; GOST P53434-2009 – "Good laboratory practice principles"; International Guiding Principles for Biomedical Research Involving Animals (1985); European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes; Guide for the Care and Use of Laboratory Animals; Decree of the Ministry of Health of the USSR №. 755 dated August 12, 1977 "About measures on further improvement of the work organizational forms, involving the experimental animals") and based on the Ethics Committee report of Federal Research Center For Virology And Microbiology in animals study (№. 12 dated February 04, 2019).

RESULTS

In order to effectively use vaccination of farm animals in combination with immunostimulating drugs, it is necessary to know how they affect the functional activity of T - and B-lymphocytes. Among the most extensively studied immunostimulating drugs in our country there can be named Glycopid (GMDP), Ribotan (5, 6). The immunostimulatory activity of Glycopid and Ribotan was investi-

gated *in vitro* via blast-transformation reaction. Peripheral blood was taken from gilts, the lymphocytes were isolated and the effect of immunostimulators in concentrations from 0.01 to 1.0 μg/cm^3, mitogens from Phaseolus vulgaris (PHA, T-mitogen) and Phytolacca americana (FA, B-mitogen), immunostimulators in the presence of mitogens were determined by the inclusion of 3H-thymidine (Table 1).

Table 1: Indexes of stimulation of blood lymphocyte proliferation in the presence of mitogens and immunostimulators, n=12

Tests	Concentration of the drug mcg / cm^3	Indexes of stimulation	
		Glycopid	Ribotan
With no mitogens	0.01	1,640,43	1,45 ± 0,33
	0.1	1,520,22*	2,15 ± 0,12*
	1.0	1,130,08	2,83 ± 0,33*
PHA	0.00	21,35 ± 1,85	22,84 ± 2,48
	0.01	20,64 ± 3,12	21.87 ± 2.55
	0.1	24,66 ± 3,82	23.99 ± 4.08
	1.0	20.08 ± 1.44	19.98 ± 2.40
FA	0.00	2,060,22	1,60 ± 0,10
	0.01	4,860,52*	1,94 ± 0,43
	0.1	4,180,35*	4,48 ± 0,46*
	1.0	3,390.23*	3,60 ± 0,92*

Note: * - credible increase at level of significance p ≤0.05

The results presented in Table 1 indicate that Glycopide and Ribotan stimulate in vitro proliferation of lymphocytes, mostly B-lymphocytes.

The immunostimulating activity of Glycopid, Immunofan, Prestimol, Ribotan and Fosprenil preparations were studied in the experiment on laboratory animals, more precisely on guinea pigs. Guinea pigs were divided into 6 groups of 4 heads; single doses of the Teschen disease vaccine were injected intramuscularly to individuals of each group into hind right paw. On the same day, one of the above immunostimulants at the dose recommended in the instructions was injected animals of each of the six experimental groups into the left hind paw. The control group was injected with buffered saline (PBS). After 21 days, the animals from all groups were anaesthetized by intraperitoneal administration of anesthetics at 100 mg xylazine and 100 mg ketamine/kg body weight, than they were bled by heart punctures. The obtained serums from guinea pigs were investigated in a reaction of neutralization. The results are presented in a Table 2.

Table 2: The effect of immunostimulants on the immunogenicity of the vaccine against Teschen disease.

Drug	Dose\head	Antibody titer in pH, average values (minimum and maximum values)
PBS	0,1 cm^3	1:448 (1:256-1:512)
Glycopid	0,05 mg	1:2048 (1024-1:4096)
Immunofan	0,1 cm^3	1:2048 (1:2048)
Prestimol	250 units	1:2730 (1:2048-1:4096)
Ribotan	0,2 cm^3	1:4096 (1:4096)
Fosprenil	0,01 cm^3	1:1024 (1:1024)

Among the studied drugs, Ribotan showed the greatest efficiency, then in descending order Prestimol, Glycopid, Immunofan, Fosprenil. The use of immunostimulating drugs combined with an inactivated vaccine increased the titers of virus-neutralizing antibodies to the Teschen disease virus in 2–9 times as compared with the control.

The effectiveness of Immunostimulants was confirmed in the experiment on pigs. Two pigs inoculated with vaccine against the Teschen disease with simultaneous introduction of Glycopide or Ribotan according to the instructions for use, the titers of viral neutralizing antibodies were 1:512-1:1024, whereas in animals of the control group vaccinated without an immunostimulator – 1:256.

The efficacy of GMDP and Ribotan has been investigated with the cattle IRT vaccine in rabbits. Three groups of rabbits weighing 2.5-3.0 kg were injected once intramuscularly with one dose of hydroxyaluminum vaccine against cattle IRT. Simultaneously, the animals of the first group were injected in the volume of 1 cm3 FBI, the second-100 mcg/kg of Glycopid, the third-5 mg/kg of Ribotan. Blood serums obtained before and on 7, 14, 21 days after vaccination were examined in PH (Table 3).

Table 3: Effect of immunostimulators on titers of virus neutralizing antibodies in rabbits after administration of vaccine against IRT cattle

№	Drug	Antibody titers in PH at different times after vaccination, days			
		0	8	14	21
1	PBS	0	1:2	1:8	1:8
2		0	1:2	1:8	1:10
3		0	1:2	1:8	1:8
4	Glycopid	0	1:2	1:8	1:10
5		0	1:2	1:8	1:10
6		0	1:2	1:8	1:8
7	Ribotan	0	1:2	1:40	1:48
8		0	1:4	1:48	1:48
9		0	1:4	1:48	1:48

The use of Ribotan led not only to higher titers of virus-neutralizing antibodies for 21 days compared to the control group and vaccination with Glycopid, but also to the formation of protective titers against IRT of cattle for 14 days after vaccination.

DISCUSSION

Currently, immunostimulants are practically not used in vaccine prophylaxis. Apparently, it is caused by various circumstances: a certain conservatism of manufacturers and users, insufficient experimental justification of expediency of use of immunostimulants, the lack of the corresponding scientific and technical documentation, the high price of immunostimulants.

We tried to experimentally substantiate the feasibility of using a number of immunostimulants together with inactivated vaccines. Immunostimulants were studied both in vivo and in vitro. The main attention was focused on the drugs GMDP and Ribotan. Moreover, the list of the studied drugs included the registered and marketed drugs Immunofan, Prestimol, Fosprenil, due to insufficient information on the effectiveness of these immunostimulants in vaccine prevention.

The intensity of lymphocyte proliferation ultimately determines the final number of specific lymphocytes with viral antigens. This, in turn, directly affects the titers of antiviral antibodies, as well as the duration of specific protection. That is why the drugs were previously investigated in RBTL, determining their ability to non-specifically activate lymphocyte proliferation. The results indicated the ability of GMDP and Ribotan to stimulate lymphocyte proliferation. A significant increase in stimulation indices was noted at drug concentrations from 0.01 to 0.10 μg/cm^3.

Ribotan drug had the best immunostimulating properties. Its simultaneous administration to rabbits with a vaccine against cattle IRT led to the achievement of protective titers as early as 7 days. The immunostimulating properties of GMDP and Ribotan were also confirmed in experiments on pigs with a vaccine against Teschen disease.

In vivo studies results of the immunostimulants effect on the immunogenicity of inactivated vaccines are interesting, in our opinion, by the fact that they were compared to each other simultaneously. The best results were obtained with Ribotan: simultaneous administration of the recommended dose of the drug with the vaccine led to an increase in titers of virus-neutralizing antibodies to the Teschen disease virus by 4-8 times.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORS CONTRIBUTION

ADS, VVM had the original idea for the study and carried out the design. AAS, LAG gathered the bibliographic material. VIK, NVS collected the samples. VNZ was responsible for data analysis and data cleaning. NPS drafted the manuscript and guided its production. The final draft manuscript was revised by all authors. All authors edited, read, and approved the final manuscript.

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