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Preclinical study of immunogenicity of adjuvanted quadrivalent subunit influenza vaccine

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Abstract

Background. Preventive vaccination is a vitally important strategic aspect of protection of the population against severe effects of influenza epidemics. The priority attention is given to development of effective tetravalent vaccines containing antigens of two influenza A lineages (H1N1, H3N2) and two influenza B lineages (Victoria and Yamagata) in combination with immunoadjuvants.

The **aim** of the work was to conduct the preclinical study of the immunogenicity and protective efficacy of the innovative tetravalent subunit vaccine containing antigens of influenza A and B viruses as well as a corpuscular adjuvant.

Materials and methods. The study was conducted using female BALB/c mice. The tetravalent vaccine and monovalent intermediate vaccines combined with a betulin adjuvant were injected intraperitoneally two times at a 14-day interval. The immunogenic activity was measured by the hemagglutination inhibition assay. The protective activity of the vaccine was assessed by changes in the viral load, body weight and survival rates using the mouse model of fatal influenza A H1N1 virus infection.

Results. The mice vaccinated with the adjuvanted quadrivalent subunit influenza vaccine produced antibodies against all four influenza viruses included in the vaccine; the mean antibody titers in the hemagglutination inhibition assay were above 1 : 40. The second-dose vaccination induced a significant increase in levels of antibodies against all four influenza viruses. The dose of the quadrivalent subunit adjuvanted vaccine containing 5 μg of each antigen and 200 μg of the adjuvant provided a 100% survival rate in mice and significantly decreased lung viral titers (more than 3 lg $TCID_{50}$) in the mouse model of influenza pneumonia.

Conclusion. The quadrivalent subunit vaccine with the betulin-based corpuscular adjuvant demonstrates high immunogenicity in laboratory mice and provides protection against fatal pneumonia caused by the influenza A virus subtype H1N1.

Keywords: *adjuvanted quadrivalent subunit vaccine, preclinical studies, influenza virus, tetravalent vaccine, adjuvant*

Ethics approval. Authors confirm compliance with institutional and national standards for the use of laboratory animals in accordance with «Consensus Author Guidelines for Animal Use» (IAVES, 23 July 2010). The research protocol was approved by the Ethics Committee of the I.I. Mechnikov Research Institute for Vaccines and Sera (protocol No. 6, April 2, 2018).

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Conflict of interest. The authors declare no apparent or potential conflicts of interest related to the publication of this article.

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ОРИГИНАЛЬНЫЕ ИССЛЕДОВАНИЯ

Научная статья
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Доклиническое изучение иммуногенности четырёхвалентной субъединичной противогриппозной вакцины, содержащей корпускулярный адъювант

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Аннотация

Актуальность. Вакцинопрофилактика является важным стратегическим аспектом защиты населения от тяжёлых последствий эпидемий гриппа. Актуальна разработка эффективных тетравалентных вакцин, содержащих антигены двух линий гриппа А (H1N1, H3N2) и двух линий гриппа В (Виктория, Ямагата) с добавлением иммуноадъюванта.

Целью работы явилось доклиническое изучение иммуногенности и защитной эффективности инновационного препарата — тетравалентной субъединичной вакцины, содержащей антигены вирусов гриппа А и В, а также корпускулярный адъювант.

Материалы и методы. Исследования выполнены на мышах-самках линии BALB/c. Тетравалентную вакцину и моновалентные полуфабрикаты с бетулиновым адъювантом вводили внутрибрюшинно двукратно с интервалом 14 дней. Иммуногенную активность оценивали по реакции торможения гемагглютинации. Протективную активность вакцины оценивали по изменению вирусной нагрузки, массы тела и выживаемости животных на модели летальной инфекции, вызванной вирусом гриппа А подтипа H1N1.

Результаты. У мышей, вакцинированных четырёхвалентной субъединичной противогриппозной вакциной с корпускулярным адъювантом, наблюдалось образование антител в отношении всех четырех вирусов гриппа, входящих в состав вакцины, средние титры антител в реакции торможения гемагглютинации были выше 1 : 40. В результате второй вакцинации наблюдался выраженный прирост антител в отношении всех четырех вирусов гриппа. Доза четырёхвалентной субъединичной вакцины с корпускулярным адъювантом, содержащая по 5 мкг каждого антигена и 200 мкг адъюванта, обеспечивала 100% выживаемость мышей, а также во всех изученных дозах значительно снижала титр вируса в лёгких у животных (более 3 lg $ТЦИД_{50}$) в модели гриппозной пневмонии.

Заключение. Четырёхвалентная субъединичная вакцина с корпускулярным адъювантом на основе бетулина демонстрирует высокую иммуногенность у лабораторных мышей и обеспечивает защиту от летальной пневмонии, вызванной вирусом гриппа А подтипа H1N1.

Ключевые слова: *четырёхвалентная субъединичная вакцина с корпускулярным адъювантом, доклинические исследования, вирус гриппа, тетравалентная вакцина, адъювант*

Этическое утверждение. Авторы подтверждают соблюдение институциональных и национальных стандартов по использованию лабораторных животных в соответствии с «Consensus Author Guidelines for Animal Use» (IAVES, 23.07.2010). Протокол исследования одобрен Этическим комитетом НИИ вакцин и сывороток им. И.И. Мечникова (протокол № 6 от 02.04.2018).

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Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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Introduction

Limitations of influenza antiviral treatment (emergence of resistant strains, safety concerns) shift the emphasis to preventive vaccination as the best method for control of influenza [1]. In the meantime, the inactivated influenza vaccines, including seasonal trivalent vaccines used for routine annual vaccination against influenza during the autumn-winter period are not always effective for a number of population groups: younger children, pregnant women, older people, people with different chronic diseases, i.e. those who are at high risk of contracting the flu, and are ineffective against antigenically different (drifted and heterologous) strains absent in the vaccine [2–4]. In addition, the production capabilities of the influenza vaccine manufacturers may not be sufficient to provide large-scale vaccination coverage, especially during a pandemic.

Quadrivalent vaccines (QV) containing 2 influenza A virus lineages (H1N1, H3N2), 2 influenza B virus lineages (Yamagata and Victoria) [5, 6], and immunoadjuvants [7] have been offered to increase the efficacy of inactivated influenza vaccines. Adjuvants enhance the immunogenicity of influenza vaccines against antigenically distinct strains and can be highly effective for immunization of different groups of population, including high-risk groups [8].

Currently, aiming to increase the immunogenicity of vaccines, experimental and clinical studies are focused on testing adjuvants of different origin:

- mineral (aluminum hydroxide or phosphate);
- plant-derived (saponins – QuilA, QS21);
- microbial (whole killed bacteria, purified bacterial lipopolysaccharide and its derivatives, CpG motifs in DNA);
- synthetic polymeric (polyoxidonium, sovidon) [9, 10].

Birch-bark triterpenoid-based adjuvants offer a promising solution in this area [11]. Natural betulin-based compounds have a wide spectrum of biological activities (antimicrobial, antifungal, antiviral, hepatoprotective, antioxidative) and, in addition to biosafety, have adjuvant properties [12].

The **aim** of the work was to conduct preclinical study of the immunogenicity and protective efficacy of the innovative tetravalent subunit vaccine containing antigens of influenza A (H1N1, H3N2) and B (Victoria, Yamagata) viruses as well as a natural betulin-based corpuscular adjuvant (CA).

Materials and methods

The tests were performed using the tetravalent subunit influenza vaccine containing the natural betulin-based adjuvant, suspension for intramuscular injection manufactured by the Institute of New Medical Technologies, CJSC.

0.5 mL of the vaccine for clinical use contains:

- 5 μg of the antigen of the A/Michigan/45/2015 (H1N1)pdm09 strain;
- 5 μg of the antigen of the A/Hong-Kong/4801/2014 (H3N2) strain;
- 5 μg of the antigen of the B/Brisbane/60/2008 strain;
- 5 μg of the antigen of the B/Phuket/3073/2013 strain;
- 200 μg of CA;
- phosphate-buffered saline (PBS) up to 0.5 mL.

We used CA (pilot batch A-1; the Institute of New Medical Technologies, CJSC) diluted with PBS. The tests were performed using adjuvant at concentrations of 1,000 and 200 μg.

The 0.5 mL monovalent samples contained:

- 5 μg of the antigen of each strain present in the vaccine sample combined with CA;
- 200 μg of CA;
- PBS up to 0.5 mL.

PBS containing the adjuvant at a concentration of 400 μg/mL was used as a placebo.

Evaluation of antigen-specific humoral immunity

The antigen-specific humoral immunity and protective efficacy of the vaccine were assessed at the Mechnikov Research Institute of Vaccines and Sera in compliance with the ethical standards and requirements[1], Good Preclinical Practice[2], Guidance on Preclinical Studies of Pharmaceuticals[3].

The tests were performed using female BALB/c mice weighing 12-14 g from the breeding farm of the Andreevka Scientific Center of Biomedical Technologies of the Russian Academy of Medical Sciences (Moscow Region). The animals were divided into 6 groups, each containing 12 mice. The vaccine (the 1st group) and monovalent samples (A/Michigan/45/2015 (H1N1) — the 2nd group; A/Hong-Kong/4801/2014 (H3N2) — the 3rd group; B/Brisbane/60/2008 — the 4th group; B/Phuket/3073/2013 — the 5th group) present in the vaccine, combined with CA, were injected at a dose of 0.25 mL to mice intraperitoneally two times at a 14 day interval. The animals from the 6th control group, instead of vaccines, were injected with 0.25 mL of the

[1] Directive 2010/63/EU of the European Parliament and the Council of 22/9/2010 on the protection of animals used for scientific purposes.

[2] International council for harmonization of technical requirements for pharmaceuticals for human use. Guideline for good clinical practice. URL: https://ichgcp.ru; GOST 22044-2014 — The Principles of Good Laboratory Practice, Sanitary and Epidemiological Requirements for Organization, Equipment, and Maintenance of Experimental Biological Clinics (Vivaria) (approved by Decree No. 51 of the Chief State Sanitary Doctor of the Russian Federation, 29/8/2014), Order No. 199-n On Approval of Rules for Good Laboratory Practice, the Ministry of Health of the Russian Federation, 1/4/2016.

[3] Guidance on Preclinical Studies of Pharmaceuticals. Moscow, 2012.

sterile placebo solution intraperitoneally on the respective days.

On the 14th day after the 1st immunization, blood samples were collected from 6 mice in each group; the other mice, except for the mice from the control group, were re-immunized intraperitoneally with 0.25 mL of the respective vaccines or saline solution. Blood sera from each of 6 animals were collected in the groups on the 14th day after the 2nd immunization. The classical hemagglutination inhibition method was used to measure titers of specific antibodies in sera from the immunized animals.

Study of protective efficacy of the vaccine

Protective properties of influenza QV combined with the betulin-based CA were studied using female BALB/c mice weighing 12–14 g from the breeding farm of the Andreevka Scientific Center of Biomedical Technologies of the Russian Academy of Medical Sciences (Moscow Region).

The vaccines were tested using the following doses:

1) QV combined with CA and containing 0.5 μg of each antigen, 200 μg of CA – 0.1 of the vaccine dose;
2) QV combined with CA and containing 5 μg of each antigen, 200 μg of CA – 1 vaccine dose;
3) QV combined with CA and containing 25 μg of each antigen, 200 μg of CA – 5 vaccine doses;
4) CA, 200 μg (the total dose of 2 immunizations);
5) CA, 1,000 μg (the total dose of 2 immunizations);
6) monovalent influenza A (H1N1) virus vaccine at a dose of 5 μg;
7) placebo (PBS, 200 mg of CA).

All the animals were immunized with 0.25 mL of the prepared vaccines intraperitoneally, two times at a 14-day interval. Instead of vaccines, the animals from the control group were injected intraperitoneally with 0.25 mL of sterile placebo solution on the respective days.

To model the influenza infection, we used the mouse-adapted influenza A/California/04/2009 strain (the 2009 H1N1 pandemic). 14 days after the 2nd immunization, the mice (18 mice in each group) were infected with the mouse-adapted influenza A/California/04/09 (H1N1) virus at a dose of 100 LD_{50} per mouse. The mice were infected intranasally with 50 μl of the virus-containing allantoic fluid under slight ether anesthesia. The animals were monitored daily for 16 days; the mice were weighed every day during 5 days following the infection and then — every other day.

The protective activity of the vaccine samples was assessed on the influenza pneumonia mouse model by three criteria:

- mortality in the groups of immunized and control mice;
- detection of the virus in the lungs of the immunized and control mice;
- weight loss in the animals.

The weight decrease or increase was calculated for each mouse and expressed as a percentage. The animals' weight before the infection was set as 100%. The average weight loss or gain percentage was calculated for all mice of each group.

Measuring lung viral titers in mice

On the 4th day following the infection with the influenza virus, 5 mice from each group were euthanized; their lungs were excised under sterile conditions. After they were lavaged three times with 0.01 M PBS, the lungs were homogenized and resuspended in 1 mL of cold sterile PBS. To remove cell debris, the suspension was centrifuged at 2,000*g* for 10 min; the supernatant was used to measure the infectious viral titer in MDCK cells.

To measure the infectious viral titer, MDCK cells were seeded in 96-well plates at the average density of 30–35 thousand cells per well and were grown in the minimum essential medium (MEM) supplemented with 5% fetal calf serum, 10 mM glutamine and antibiotics (100 IU/mL penicillin and 100 μg/mL streptomycin) till they grew to a complete monolayer. Before they were infected with the virus, the cells had been washed 2 times with serum-free MEM. Ten-fold serial dilutions of each virus sample from lungs (up to 10-8) were prepared in the medium by adding TPCK trypsin (2 μg/mL). The dilutions were used to infect the monolayer of 4 wells in the 96-well plate. After the 72-hour incubation at 37°C and 5% CO_2, the cells were washed with PBS three times and fixed with 10% formaldehyde solution at 18–23ºC for 5 min. After the formaldehyde solution was removed, 100 μl of 1% crystal violet solution was added to each well in the plate and was left there at 18–23ºC for 5 min. After the plate was washed with water and dried, 0.1 mL of 96% alcohol was added to the wells, incubated with shaking at room temperature for 20 min; then the optical density was measured at the 570 nm wavelength. The wells were deemed to have positive results, if the optical density in them was less than the optical density in the cell control wells by 20%. The infectious viral titer was measured using the Reed and Muench method, 4 times in each sample and expressed as log10 $TCID_{50}$/0.1 mL (TCID – tissue culture infectious dose). Then, the average value of the titer was calculated for 3 identical samples.

Statistical analysis of the results

The statistical analysis was performed using descriptive statistics methods. The titers of specific antibodies are presented as geometric means and their confidence intervals. The distribution normality for variables in the groups was checked using the Shapiro–Wilk test. When variables were not normally dis-

tributed ($p < 0.05$), the comparison was performed using non-parametric Kruskal–Wallis and Mann–Whitney tests. When the data showed normal distribution ($p \geq 0.05$), the comparison was performed using the analysis of variance (ANOVA) and Dunnett's test. The pairwise comparison of the groups was performed using the Student t-test. Differences between the groups were considered statistically significant at $p \leq 0.05$.

Results

The study of the influenza QV combined with natural betulin-based CA included assessment of the antigen-specific humoral immunity as well as assessment of protective properties of the vaccine against the influenza A /California /04/09 (H1N1) virus using the animal influenza pneumonia model.

Assessment of antigen-specific humoral immunity

Prior to the immunization, no specific antibodies with detectable levels were found by the tests.

The measurement results for the geometric mean titers of hemagglutination inhibition antibodies in mice immunized with the studied samples are presented in **Table 1**. It was found that the control group animals immunized with placebo solution instead of vaccines did not have antibodies.

The group immunized with QV combined with CA demonstrated production of antibodies against all four influenza A and B viruses present in the vaccine (Table 1). In the hemagglutination inhibition assay, the mean antibody titers were higher than 1:40 for all tested influenza viruses, though antibody titers against influenza B viruses were lower than those against influenza A viruses. The mean antibody titers against the respective viruses, which were induced by the vaccination with the monovalent A/California/07/09 (H1N1) vaccine as well as with antigens of influenza A H3N2 and influenza B viruses, were higher than 1:40. Like with QV, antibody titers against influenza B viruses were lower than those against influenza A viruses. The highest antibody responce after the 1st immunization was observed against the influenza A/California/07/09 (H1N1) virus.

The second immunization was conducted 14 days after the first one. Sera from 6 animals from each group were collected on the 14th day after the 2nd immunization. During the entire period after the 2nd immunization, the animals were also monitored; no deviation in the behavior of the animals was detected; the animals had no evidence of the disease; none of the mice died. The 6th group did not demonstrate any increase in antibody levels. The other groups demonstrated production of antibodies against the respective viruses; after the 2nd vaccination, the antibody titers increased significantly compared with the titers after the 1st vaccination, except for titers in the animals vaccinated with the monovalent Yamagata virus vaccine. As it was observed after the 1st vaccination, the antibody titers against influenza A viruses were higher than antibody titers against the influenza B virus, when using both QV and the monovalent vaccine components. Both after the 1st vaccination and after the 2nd vaccination, the highest increase in the antibody titers in the blood of the animals was detected against the A/California/07/09 (H1N1) virus.

Assessment of the protective efficacy of the vaccine

The mice from the 6th group started to die on the 4th day after they had been infected, and by the 12th day of the monitoring period, all the animals had died (**Fig. 1**). In the groups of animals who received CA at a dose of 200 and 1,000 μg, respectively, the death rates were slightly lower – 85% and 77%. The protective activity of QV combined with CA was dose-dependent. The

Table 1. Immunogenicity of CA-containing QV and its component after the 1st and 2nd immunizations of mice (the level of influenza virus-specific antibodies in laboratory animals after the immunization), geometric mean titers [95% confidence interval]

Group	Influenza virus strain							
	1st immunization				2nd immunization			
	H1N1	H3N2	Victoria	Yamagata	H1N1	H3N2	Victoria	Yamagata
1	142,5 [105,9–191,8]	100,8 [69,2–146,7]	56,6 [38,0–84,2]	80,0 [50,5–126,7]	1140,4 [847,4–1534,5]	640,0 [404,0–1013,8]	359,2 [266,9–483,4]*	201,6 [111,3–365,0]*
2	100,8 [69,2–146,7]	–	–	–	806,3 [553,9–1173,9]	–	–	–
3	–	80,0 [50,5–126,7]	–	–	–	403,2 [167,1–972,7]	–	–
4	–	–	44,9 [33,4–60,4]	–	–	–	226,3 [151,9–337,0]	–
5	–	–		50,4 [34,6–73,4]	–	–	–	71,3 [34,9–145,7]
6	–	–	–	–	–	–	–	–

Note. *$p < 0.05$ compared to the respective monovalent vaccine (the Student *t*-test).

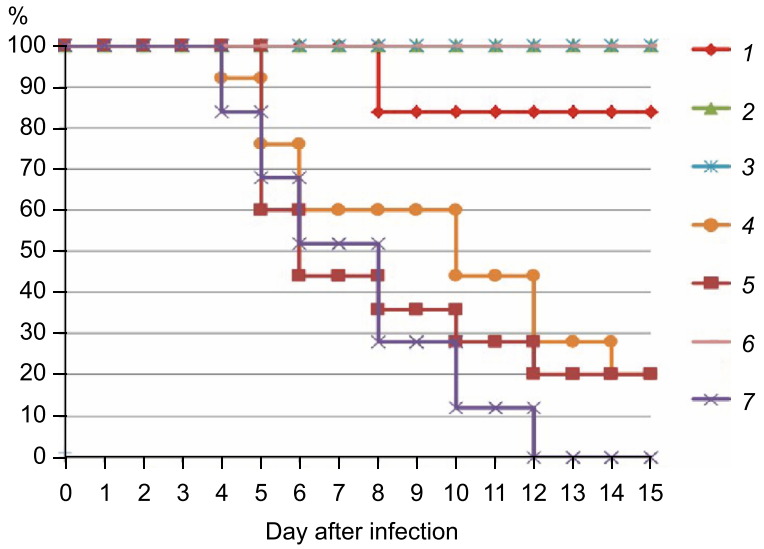

Fig. 1. Survival rates in animals vaccinated with the influenza CA-containing QV or with CA at different doses the challenge infection with the influenza A/California/04/09 (H1N1) virus.

1 — 0.1 of the vaccine dose; *2* — 1 vaccine dose; *3* — 5 vaccine doses; *4* — CA, 200 μg; *5* — CA, 1,000 μg; *6* — the monovalent H1N1 vaccine, 5 μg; *7* — control.
In all cases, $p < 0.0001$ compared to the control group.

animals immunized with the 0.1 vaccine dose demonstrated the 85% survival rate. All the animals immunized with 1 and 5 vaccine doses survived.

The vaccination with the monovalent vaccine containing the antigen of the influenza A/California/04/09 (H1N1) virus at a dose equivalent to its content in QV combined with CA also protected all the infected animals against death. The data on the death rates in the groups of animals completely correlated with the data on changes in the body weight of animals from these groups (**Fig. 2**). In the control unvaccinated group, the weight losses were the highest after the challenge infection and reached 34.3% on the 10th day. In the group of animals who received CA, the weight losses were slightly lower compared to the control group, however, these differences were not statistically significant. In the group of animals immunized with 0.1 of the vaccine dose, the weight loss was significantly lower than in the control group. In the other groups with no death cases among the animals, weight losses after the infection were insignificant and were not significantly different from the weight losses in the control group.

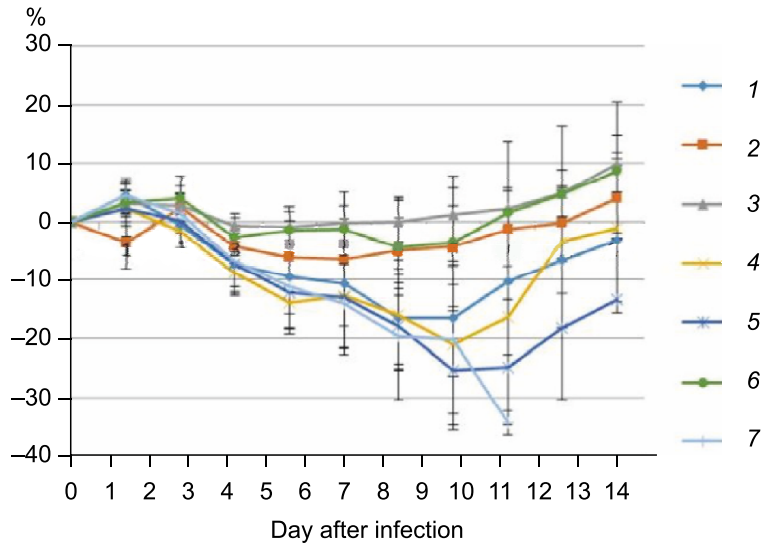

Fig. 2. Changes in the body weight of animals vaccinated with the CA-containing influenza QV and its components after the challenge infection with the influenza A/California/04/06 (H1N1) virus.

1 — 0.1 of the vaccine dose ($p = 0.391$); *2* — 1 vaccine dose ($p = 0.00398$); *3* — 5 vaccine doses ($p = 0.00391$); *4* — CA, 200 μg ($p = 0.993$); *5* — CA, 1000 μg ($p = 0.503$); *6* — monovalent vaccine H1N1, 5 μg ($p = 0.00398$); *7* — control.

Lung viral titers in mice

We analyzed the effect of QV combined with CA at different doses on lung viral titers of the infected mice. The titers were measured on the 4th day after the challenge infection. The data of the virological analysis of the lungs completely correlated with the data on protective activity of the tested samples (**Table 2**).

In the control group of unvaccinated animals, the lung viral titers reached the highest levels of 5.8 ± 0.45 lg $TCID_{50}$, being indicative of the rapid progression of the infection that resulted in the death of the animals. In the groups of animals who received only CA, the viral titers were almost similar to the lung viral titers in the animals from the control group. The lung viral titers in the animals vaccinated with QV combined with CA depended on the dose of antigens and in all cases were significantly different from the lung viral titers in the animals from the placebo control group. The highest viral titer (4.2 ± 0.45 lg $TCID_{50}$) was detected in the animals immunized with 0.1 of the vaccine dose; as expected, the increasing vaccine doses resulted in the decreasing viral titers that reached their lowest value (1.2 ± 1.09 lg $TCID_{50}$) in the group of animals immunized with 5 vaccine doses. In the animals vaccinated with the vaccine mono-component containing the antigen of the influenza A/California/04/09 (H1N1) virus, after the challenge infection with the same virus, the lung viral titers (2.4 ± 0.55) in the animals were almost similar to the viral titers in the group of animals vaccinated with QV combined with CA at the same dose, though were significantly lower than the viral titers in the control group.

Discussion

On the whole, the results of the study are consistent with the data published by other authors reporting high efficacy and safety of influenza vaccines.

The preclinical studies demonstrated that QV combined with CA is characterized by high protective activity, significantly decreasing death rates or completely protecting animals against death, and reducing weight losses compared to the control group after the lethal challenge; the vaccine dose containing 5 μg of each antigen and 200 μg of CA is sufficiently effective and provides a 100% survival rate in the experimental animals infected with the influenza virus. It should be noted that the vaccine demonstrates its effectiveness at a reduced antigen load, 5 μg of the antigen against 15 μg used in the licensed split influenza vaccines such as polymer-subunit Grippol Plus, subunit Influvac, and split-virion Vaxigrip [12].

At all the studied doses, QV combined with CA significantly decreases lung viral titers in animals (more than 3 lg $TCID_{50}$) after the lethal challenge.

The projected favorable safety profile was comparable with the profile of the licensed vaccines.

Conclusion

Thus, QV combined with the betulin-based CA demonstrates high immunogenicity in laboratory mice and provides protection against fatal pneumonia caused by the influenza A virus, subtype H1N1. The positive results of the preclinical study of the immunogenicity and protective efficacy of the innovative CA-containing influenza QV against H1N1 offer solid grounds for recommending further preclinical and clinical trials of the vaccine for its future use in preventive vaccination.

Table 2. Lung viral titers in animals vaccinated with QV combined with CA and infected with the influenza A/California/04/09 (H1N1) virus

Group	Survival rate, %	Lung viral titers, lg $TCID_{50}$
0.1 of the vaccine dose	85	4,2 ± 0,45
1 vaccine dose	100	2,4 ± 0,89
5 vaccine doses	100	1,2 ± 1,09
CA 200 μg	15	5,3 ± 0,84
CA 1000 μg	23	5,8 ± 0,27
Monovalent H1N1 vaccine	100	2,4 ± 0,55
Control	0	5,8 ± 0,45

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