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Modeling of mixed infection with Zika and West Nile viruses (*Flaviviridae: Orthoflavivirus: Orthoflavivirus zikaense, Orthoflavivirus nilense*) *in vitro* and *in vivo*

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Abstract

Introduction. Mosquito-borne human diseases caused by Zika virus and West Nile virus (WNV) are widespread across multiple continents and cause major outbreaks. Their ranges overlap and the possibility of mixed infections is obvious. The information of such mixed infections is limited.

The aim of the study is to investigate the features of mixed infection of WNV and Zika virus *in vitro* and *in vivo* in order to assess their possible interference and/or enhancement of viral infection.

Materials and methods. The study used West Nile virus and Zika virus strains Vlg27924 and MR766, respectively. The infectious activity of viruses during mono- and co-infection was determined on Vero E6 cell culture using RT-PCR, as well as on BALB/c mice using various administration schemes.

Results. *In vitro* studies of co-infection with WNV and Zika virus showed that co-infection leads to interference, with the degree of competitive inhibition of replication being more pronounced for Zika virus, reaching 1000 times or more when compared to mono-infection. During simultaneous infection in mice, Zika virus does not affect the development of lethal infection caused by WNV. However, preliminary (4 and 20 days) infection with a sublethal dose of Zika virus reliably protects animals from subsequent administration of 10 and 100 LD₅₀ WNV, respectively. In pre-infected and co-infected animals with Zika virus, the development of WNV-specific viral neutralizing antibodies was recorded in higher titers than in WNV monoinfected animals.

Conclusion. The presence of *in vitro* interference between the studied orthoflaviviruses was shown, most pronounced in relation to the Zika virus. No significant effect was observed with simultaneous co-infection *in vivo*. However, pre-infection of mice with Zika virus provides protection to animals from lethal WNV infection due to the induction of high levels of antibodies that specifically neutralize its infectious activity.

Keywords: Zika virus; West Nile virus; co-infection; viral interference

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ОРИГИНАЛЬНОЕ ИССЛЕДОВАНИЕ

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Моделирование смешанной инфекции вирусами Зика и Западного Нила (*Flaviviridae: Orthoflavivirus: Orthoflavivirus zikaense, Orthoflavivirus nilense*) *in vitro* и *in vivo*

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Резюме

Введение. Заболевания человека, вызываемые вирусами Западного Нила (ВЗН) и Зика и передающиеся через укус комара, широко распространены на разных континентах и обуславливают крупные вспышки. Ареалы этих вирусов перекрываются, что создает возможность возникновения микст-инфекций. Знания о подобных микст-инфекциях ограничены.

Цель исследования. Провести изучение особенностей протекания микст-инфекции ВЗН и вируса Зика *in vitro* и *in vivo* с целью оценки их возможной интерференции и/или усиления инфекции.

Материалы и методы. В работе использовали штаммы Влг27924 и MR766 ВЗН и вируса Зика соответственно. Инфекционную активность вирусов при моно- и коинфицировании определяли на культуре клеток Vero E6 с помощью полимеразной цепной реакции с обратной транскрипцией, а также на мышах линии BALB/c.

Результаты. При исследовании коинфицирования ВЗН и вирусом Зика *in vitro* показано, что совместное инфицирование приводит к интерференции, при этом степень конкурентного ингибирования репликации более выражена в отношении вируса Зика (в 1000 раз и более) при сравнении с моноинфекцией. При одновременном инфицировании мышей вирус Зика не влияет на развитие летальной инфекции, вызванной ВЗН. Однако предварительное (за 4 и 20 сут) инфицирование сублетальной дозой вируса Зика достоверно защищает животных от последующего введения 10 и 100 ЛД₅₀ ВЗН. У предварительно инфицированных вирусом Зика коинфицированных животных зарегистрировано появление специфических к ВЗН вируснейтрализующих антител в более высоком титре, чем у моноинфицированных ВЗН особей.

Заключение. Показано наличие интерференции *in vitro* между исследованными ортофлавивирусами, наиболее выраженной в отношении вируса Зика. При одновременном инфицировании мышей вирус Зика не влияет на течение и исход инфекционного процесса, вызванного ВЗН. Предварительное инфицирование мышей вирусом Зика обеспечивает защиту животных от летальной инфекции ВЗН, праймируя индукцию более высокого уровня вируснейтрализующих антител.

Ключевые слова: вирус Зика; вирус Западного Нила; коинфекция; интерференция

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Финансирование. Исследование было выполнено при поддержке Министерства науки и высшего образования Российской Федерации, соглашение № 075-15-2025-526.

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

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 Биоэтическим комитетом ФБУН ГНЦ ВБ «Вектор» (Протокол № 2 от 03.04.2023).

Introduction

Many RNA-containing orthoflaviviruses belonging to the Flaviviridae family (*Riboviria; Orthornavirae; Kitrinoviricota; Flasuviricetes; Amarillovirale; Flaviviridae; Orthoflavivirus*) cause severe human diseases [1]. Currently, orthoflaviviruses are widespread almost everywhere, forming natural infection foci that sometimes encompass entire regions and continents, as

well as causing global outbreaks of human diseases [2, 3]. It is customary to distinguish the so-called major flavivirus infections, which are caused by dengue, Japanese encephalitis, West Nile (WNV), yellow fever, and Zika viruses, and with which tens of millions of human infections are associated [4]. The onset of the global outbreak of West Nile fever associated with WNV genotype 1a is related with outbreaks of the disease in 1999 in the USA and Russia [5]. Over the next few years, this infection

spread to almost every continent [6–8]. The rapid spread of the infection has been linked to various species of wild birds and mosquitoes of the genus *Culex*. In the Russian Federation, WNV genotypes 1 and 2 constantly circulate in the central and southern European parts of Russia, in the south of Western Siberia and in the Far Eastern region [6, 7]. *Culex pipiens* mosquitoes, as well as *C. tarsalis* and *C. quinquefasciatus*, are considered the main vectors of WNV in various regions of the world.

Zika virus was originally isolated in Uganda in 1947 and was associated with *Aedes* mosquitoes [9]. Later, its circulation was described in countries of the Indian Ocean basin and in 1966 the Asian genotype of the Zika virus was isolated [10]. In fact, the widespread spread of Zika fever began in 2007, and in 2015–2016 the disease began to be registered in the countries of Central and South America. By the end of 2023, the infection had been reported in more than 92 countries, not counting countries, including Russia, where imported cases of the disease had been reported. The main vector for the spread of Zika fever is considered to be *Aedes aegypti* and *Ae. albopictus* mosquitoes, however, other mosquitoes of the genus *Aedes* may also be involved in the spread of the Zika virus [10].

Orthoflaviviruses, like other arboviral infection agents, can be transmitted through mosquito bites, which can be infected with several types of pathogens simultaneously [11]. Thus, *Ae. aegypti* and *Ae. albopictus* mosquitoes have been simultaneously infected with Zika, dengue and chikungunya viruses [12]. There are known cases of mixed infections with Zika/chikungunya, Zika/dengue, dengue/chikungunya and Zika/dengue/chikungunya in humans associated with mosquitoes [13–17]. The clinical picture of mixed infections caused by orthoflaviviruses may differ from mono-infections, making their diagnosis and treatment more difficult. It is important to note that for a number of mixed infections, modulation of virulence is unique to them, which can lead to an increase (or decrease) in the severity of the disease [18, 19]. Thus, sequential infection with different subtypes of the dengue virus can cause a sharp increase in the severity of the disease in humans, up to and including fatal cases [20]. This phenomenon is usually associated with the action of pre-existing antibodies to another subtype of the dengue virus, which causes an increase in the severity of recurrent dengue fever in humans.

The importance of the issue of mixed infections is due to the complexity of prevention, diagnosis, and treatment of such cases. At the same time, there is insufficient information about the occurrence, characteristics and consequences of mixed infections in different regions of the world [21].

The aim of this study is the experimental investigation of the characteristics of mixed infection with WNV and Zika virus *in vitro* and *in vivo*.

Materials and methods

The study experiments used WNV (strain Vlg27924) and Zika virus (strain MR766) obtained from the State Collection of Pathogenic Microorganisms and Rickettsiae

of the State Research Center of Virology and Biotechnology «Vector». The viruses were cultured on monolayer cell cultures of Vero E6 and SPEV (swine embryonic kidney cell culture), grown to 80–90% confluency in DMEM F12 medium (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA), penicillin 100 IU/mL, and streptomycin 100 µg/mL (Gibco, USA), in an atmosphere with 5% CO₂ at 37 °C. The infectious activity of the viral substances was determined by microtitration on 96-well culture plates (Greiner, Austria) with a subconfluent monolayer of cells, as previously described [22, 23]. The results were recorded with microscopy after 6 days based on the development of CPE (cytopathogenic effect) and/or using the MTT assay. The calculation of viral infectious titers was performed using the Spearman-Kärber method and expressed as log₁₀ TCID₅₀ (50% tissue culture infectious dose). The viral substances were stored at a temperature of –80 °C.

The study of the effects of *in vitro* co-infection was conducted using Vero E6 cells with simultaneous and sequential (in different order) infection of cell cultures with WNV and Zika virus. Infected cell cultures (three repetitions per experimental point) in 24-well culture plates (Greiner, Austria) were incubated at 37 °C in a CO₂ incubator, and samples were taken at different time intervals to determine viral load using reverse transcription polymerase chain reaction (RT-PCR) as we described earlier [24]. Mono-infected cell cultures were used as controls. RNA was extracted from samples using the Ribo-Prep RNA kit (Central Research Institute of Epidemiology of Rospotrebnadzor, Moscow). To detect viral RNA, reagent kits for identifying WNV and Zika virus RNAs (State Research Center of Virology and Biotechnology «Vector», Koltsovo, Novosibirsk region) were used by the RT-PCR method with hybridization-fluorescent detection in real-time mode, using primers specific to the 3'-UTR and NS5 gene, respectively. Specificity control was performed using viral suspensions containing 10⁶ TCID₅₀/mL of WNV or Zika virus. The absence of a significant effect of one virus on the quantitative detection of another in the mixture was confirmed by a model experiment (Fig. S1, Supplementary Materials). The registration of results was carried out using the CFX 96 device (Bio-Rad, USA). Standard curves were generated by 10-fold serial dilution of the internal positive control samples (IPCS) supplied with the respective PCR kit (from 10⁶ to 0.1 copies/reaction). Ct values of samples were obtained from two fluorescence channels, for viral cDNA and for IPCS. Viral cDNA Ct values were scaled relative to IPCS Ct values [25].

Infection of BALB/c mice weighing 10–12 g was carried out by intraperitoneal administration of 200 µL of a virus-containing suspension, while control animals were administered an equivalent volume of saline solution. In the study of mixed infections, the following experimental schemes were used: mono-infection of animals with Zika virus and WNV; simultaneous infection with Zika virus and WNV; sequential infection (Zika virus and subsequently WNV after 4 days; Zika virus and subsequently WNV after 20 days). Mono-infected animals with either WNV

or Zika virus were used as comparison. During the experiment, the condition of the animals was assessed daily. The animals were housed in same-sex groups in individually ventilated cages (Animal Care Systems) under controlled conditions, at a temperature of 22–26 °C and a relative humidity of 30–60%. Granulated feed and water were provided *ad libitum* to the animals.

The neutralization reaction (NR) of WNV or Zika virus was conducted using the micro-method on Vero E6 cells with double serial dilutions (starting from 1:10) of individual mouse sera, heated at 56 °C for 30 minutes [26]. The titers of virus-neutralizing antibodies were taken as the inverse values of the serum dilutions that completely neutralized the infectious activity of WNV or Zika virus at a dose of 50 TCID₅₀.

Statistical analysis, including calculation of mean, standard deviation, and coefficient of variation of the mean Ct value, was performed using Excel (Microsoft Corp., USA). Statistical processing of data was also carried out using the statistical program STATISTICA 12 (StatSoft Inc., USA). Statistical assessment of differences between groups was performed using Student's t-test; a p value < 0.05 was considered significant.

Experiments with infectious material were conducted in accordance with the requirements of biosafety regulations as stipulated in SanPiN 3.3686-21 "Sanitary and Epidemiological Requirements for the Prevention of Infectious Diseases" dated January 1st, 2021. Infection of mice with WNV and Zika virus along with the rest of the manipulations with them were carried out in the infectious vivarium of the State Research Center of Virology and Biotechnology «Vector» of Rospotrebnadzor under BSL-3 conditions.

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 study protocol was approved by the Bioethics Committee of the State Scientific Center of Virology and Biotechnology «Vector» of Rospotrebnadzor (Protocol No. 02 of April 03, 2023).

Results

Mixed infection *in vitro*. The replicative activity of viruses *in vitro* during mixed infection compared to mono-infection was assessed by determining viral load using quantitative PCR analysis (Table 1). Simultaneous infection led to pronounced inhibition of Zika virus replication, with no significant effect of coinfection on the accumulation of infectious viral particles in infected cells (Table 1 *a*). Sequential infection led to competitive inhibition of the viral infectivity, with the degree of competitive inhibition being significantly more pronounced for the Zika virus (Table 1 *b*). Thus, the viral load of the Zika virus in cells pre-infected with WNV and then infected with the Zika virus after 24 hours was more than 3 orders of magnitude lower than in the corresponding Zika virus mono-infected cells. In reverse experiments, the differences in viral load of WNV in cells with mixed-infection and mono-infection did not exceed 1.5 log₁₀.

Mixed infection *in vivo*. Co-infection with WNV and Zika virus was modeled through simultaneous and sequential infection of mice with different doses of the viruses (Zika virus and WNV after 4 days; Zika virus and WNV after 20 days). The results presented in Fig. 1 show that infection with the Zika virus (10⁴ TCID₅₀/mouse) did not cause the development of a lethal infection, and no visible clinical manifestations of viral infection were observed in the animals. On the contrary, infection of mice with WNV at a dose of 10³ TCID₅₀ led to the death of 90% of the mice with the development of clinical symptoms of neuroinfection. In the case of mixed simultaneous infection with two viruses, the Zika virus did not significantly affect the course and outcome of the infectious process caused by WNV. At the same time, when animals were pre-infected with the Zika virus (4 days before being infected with WNV), 80% of the mice infected with 10 LD₅₀ of WNV survived.

Figure 2 shows the results of infecting BALB/c mice 20 days after prior infection with the Zika virus at a dose of 10⁴ TCID₅₀/mouse. A 100% and 90% protection of Zika virus pre-infected animals from sequential infection with doses of 10 and 100 LD₅₀ of WNV, respectively, was observed. These data indicate that prior infection with the Zika virus provides significant protection to animals against sequential infection with lethal doses of the Zika virus.

Induction of virus-neutralizing antibodies in BALB/c mice. Individual sera from experimental animals were tested for the presence of virus-neutralizing antibodies (Table 2). Serum from mice infected with either Zika virus or WNV neutralized only homologous viruses. However, after being infected with WNV, the sera of animals previously infected with the Zika virus were able to neutralize both the Zika virus and WNV. At the same time, the titers of virus-neutralizing antibodies in sera that were pre-infected with the Zika virus and then infected with 100 LD₅₀ of WNV were significantly higher than in animals infected only with WNV. To confirm the specificity of the neutralizing antibodies and the absence of their cross-reactivity in the serum, an assessment of their neutralizing activity against a heterologous virus was conducted. Thus, the sera of surviving mice after their infection with WNV at a dose of 10 LD₅₀, 21 days post-infection, had a high titer of neutralizing antibodies against WNV (750 ± 107), but they were unable to neutralize the Zika virus in the cell culture.

Discussion

The phenomenon of interference between different viruses in a susceptible cell has been known for quite some time and, in its classical form, suggests the suppression of one virus's replication by another [27]. Possible mechanisms of this phenomenon are associated with various key points of viral replication in the cell, induction of antiviral defense systems in the cell, including interferon systems, competition for cellular resources for the synthesis of viral molecules and viral particles, different rates of viral replication, and a number of other factors. The entry of a viral particle into a cell

Table 1. Simultaneous and sequential infection of Vero E6 cell culture with West Nile virus (WNV) and Zika virus (MOI 0.5 TCID₅₀)**Таблица 1.** Одновременное и последовательное инфицирование культуры клеток Vero E6 вирусом Западного Нила (ВЗН) и вирусом Зика (МОИ 0,5 ТЦД₅₀)

Simultaneous infection (a) Одновременное инфицирование (a)			
Time post-infection Время после инфицирования	WNV (log10 RNA copies/mL) ВЗН (lg копий РНК/мл)	Zika virus (log10 RNA copies/mL) Вирус Зика (lg копий РНК/мл)	WNV/Zika virus (log10 RNA copies/mL) ВЗН/вирус Зика (lg копий РНК/мл)
24 h / ч	< 3.0	< 3.0	< 3.0/< 3.0
48 h / ч	4.3 ± 0.3	3.8 ± 0.2	4.0 ± 0.3/3.2 ± 0.2
72 h / ч	6.5 ± 0.4	5.7 ± 0.3	6.3 ± 0.4/3.5 ± 0.2
96 h / ч	8.1 ± 0.4	7.8 ± 0.3	7.7 ± 0.3/3.9 ± 0.3
Sequential infection (b) Последовательное инфицирование (б)			
Time post-infection with Zika virus Время после инфицирования вирусом Зика	WNV–24 hours–Zika virus ВЗН–24 ч–вирус Зика		PBS–24 hours–Zika virus PBS–24 ч –вирус Зика
	WNV/ Zika virus (log10 RNA copies/mL) ВЗН/вирус Зика (lg копий РНК/мл)		Zika virus (log10 RNA copies/mL) Вирус Зика (lg копий РНК/мл)
24 h / ч	4.5 ± 0.3/< 3.0		< 3.0
48 h / ч	6.2 ± 0.4/3.2 ± 0.3		4.2 ± 0.2
72 h / ч	7.4 ± 0.3/3.4 ± 0.3		6.9 ± 0.3
Time post-infection with WNV Время после инфицирования ВЗН	Zika virus–24 hours–WNV Вирус Зика–24 ч–ВЗН		PBS–24 hours–WNV PBS–24 ч–ВЗН
	Zika virus/WNV (log10 RNA copies/mL) Вирус Зика/ВЗН (lg копий/мл)		WNV (log10 RNA copies/mL) ВЗН (lg копий РНК/мл)
24 h / ч	3.5 ± 0.3/< 3.0		< 3.0
48 h / ч	5.1 ± 0.4/3.3 ± 0.3		4.2 ± 0.2
72 h / ч	6.5 ± 0.4/5.0 ± 0.3		6.4 ± 0.2

Note. Values represent $M \pm SD$ of three independent experiments. Student's t-test was used to compare two groups.**Примечание.** Значения представляют собой $M \pm SD$ трех независимых экспериментов. Для сравнения двух групп использовался *t*-критерий Стьюдента.

can dramatically alter the functioning of the cellular genome, including or inhibiting various genes [28]. These changes can involve dozens or even hundreds of cellular genes. The complexity of conducting these studies is predetermined by our insufficient knowledge of the phenomenon of interference between different viruses, and the development of research on the genetic diversity of viromes has highlighted the urgent necessity to facilitate research development in this area [29]. The peculiarity of orthoflaviviruses is that in natural foci, they are usually spread by vectors [11]. Ixodid ticks and various species of mosquitoes are the most common vectors of these viruses. In the case of overlapping natural foci, mixed infections are possible in both invertebrates and their warm-blooded hosts. The Zika virus and WNV belong to the so-called mosquito-borne flaviviruses and often circulate in the same regions, so cases of co-infection are quite likely among susceptible host species, including humans.

In the experiments of the current study, Zika virus and WNV, which are widely distributed in the modern world and have recently caused nearly global outbreaks

of diseases in countries with warm climates, were used. The results obtained using these viruses demonstrate the presence of *in vitro* interference between these two orthoflaviviruses. Simultaneous infection leads to pronounced inhibition of Zika virus replication, with no similarly pronounced effect on WNV replication in mammalian cells. Sequential (in different order) infection also leads to competitive mutual inhibition of the replication of these viruses, with the degree of competitive inhibition remaining more pronounced for the Zika virus. Recently, it was shown that coinfection of Vero cells with WNV and St. Louis encephalitis virus (SLEV) is accompanied by inhibition of SLEV replication with minimal effect on WNV replication activity. At the same time, coinfection of mosquito cells CT (*Cx. tarsalis*) did not affect the replication of any of the viruses [30].

Mixed infections in laboratory animal models (*in vivo*) partially confirmed the results obtained in the cell culture. Thus, simultaneous infection of BALB/c mice with the Zika virus did not significantly affect the course of the disease and the fatal outcome of the infectious process caused by WNV. However, when the animals were pre-

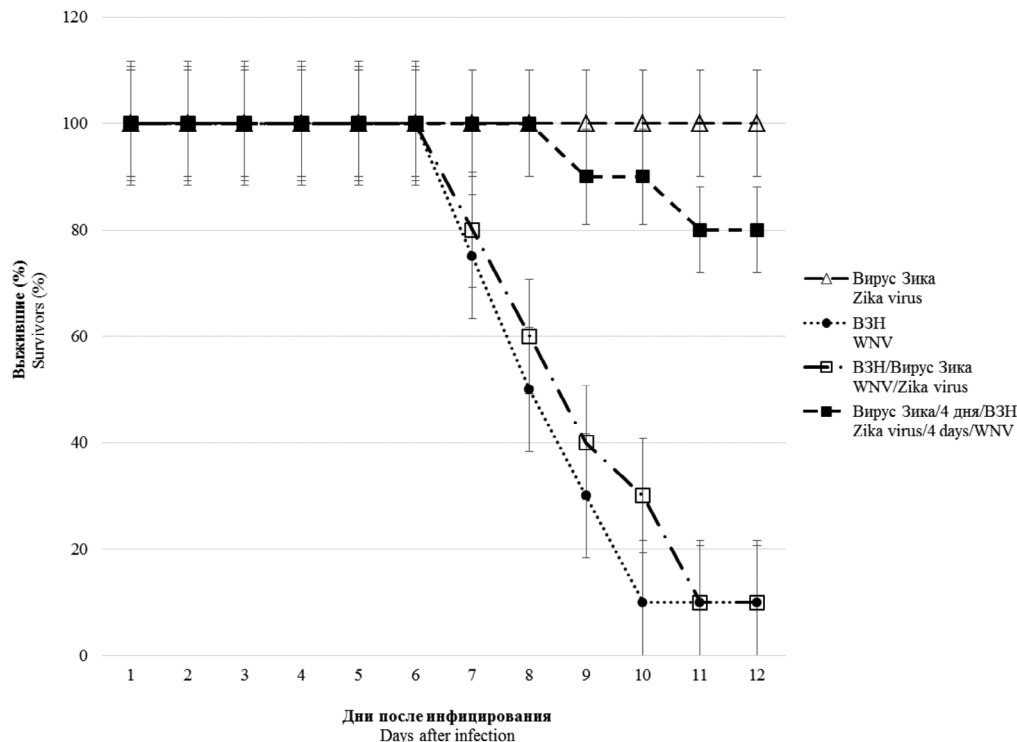


Fig. 1. Survival of BALB/c mice infected with West Nile virus (WNV) and Zika virus under different schemes of virus administration.

△ – experimental results obtained by intraperitoneal Zika virus infection with a dose of 10^4 TCID₅₀; ● – experimental results obtained by intraperitoneal infection of WNV with a dose of 10^3 TCID₅₀ (10 LD₅₀); □ – experimental results obtained by simultaneous intraperitoneal infection with Zika virus (10^4 TCID₅₀) and WNV (10 LD₅₀); ■ – experimental results obtained by intraperitoneal infection with Zika virus (10^4 TCID₅₀) and after 4 days intraperitoneal infection with 10 LD₅₀ of WNV ($n = 10$; $p < 0.05$).

Рис. 1. Выживаемость мышей BALB/c, инфицированных вирусами Западного Нила (ВЗН) и Зика, при разных схемах введения вирусных препаратов.

△ – результаты, полученные при внутрибрюшинном инфицировании вирусом Зика дозой 10^4 ТЦД₅₀; ● – при внутрибрюшинном инфицировании ВЗН дозой 10^3 ТЦД₅₀ (10 ЛД₅₀); □ – при одновременном внутрибрюшинном инфицировании вирусом Зика (10^4 ТЦД₅₀) и ВЗН (10 ЛД₅₀); ■ – при внутрибрюшинном инфицировании вирусом Зика (10^4 ТЦД₅₀) и через 4 сут внутрибрюшинном инфицировании ВЗН (10 ЛД₅₀) ($n = 10$; $p < 0,05$).

infected with the Zika virus (4 days before being infected with WNV), a significant decrease in WNV infectious activity was observed in the laboratory animals. These results indicate the possibility of *in vivo* interference between these two flaviviruses. We were unable to assess the reverse effect due to the fact that the chosen animal infection model did not ensure the development of clinically apparent and/or lethal infection after the administration of the Zika virus. When modeling co-infection by infecting animals with WNV 20 days after Zika virus infection, it was found that prior infection with Zika virus almost completely protects the animals from subsequent infection with 10 and 100 LD₅₀ of WNV.

In the current study, it was demonstrated that, unlike previously published data on mixed infections with Zika and dengue viruses [30, 31], WNV infection in mice pre-infected with Zika virus was not accompanied by an exacerbation of infection symptoms. Moreover, upon prior infection with the Zika virus, the development of significant protection in animals against subsequent infection with WNV was observed. The key role of neutralizing antibodies in protection against flavivirus infections has been previously demonstrated [32–34]. The assessment of

the ability of serum samples to neutralize Zika virus and WNV was tested in a viral neutralization assay on Vero E6 cell culture. The induction of neutralizing antibodies was observed only against the homologous virus (Table 2), and cross-reactivity of neutralizing antibodies was absent. It is important to emphasize that prior infection with the Zika virus led to a more rapid response to WNV infection, enhancing the subsequent production of neutralizing antibodies against WNV. Moreover, the level of antibodies was sufficient to protect the animals from lethal infection. The ambiguity of potential outcomes of mixed infection with WNV and Zika virus is illustrated in [35], which showed that serum samples from WNV-seropositive individuals effectively neutralize WNV but enhance the infectious activity of Zika virus.

The biological significance of a prior non-lethal Zika virus infection, which provides protection against WNV, may limit the circulation of these orthoflaviviruses in the natural foci of these infections. These results may be useful for further studying mixed infections caused by Zika and WNV, as well as for the future development of broad-spectrum immunoprophylactic agents against flavivirus infections.

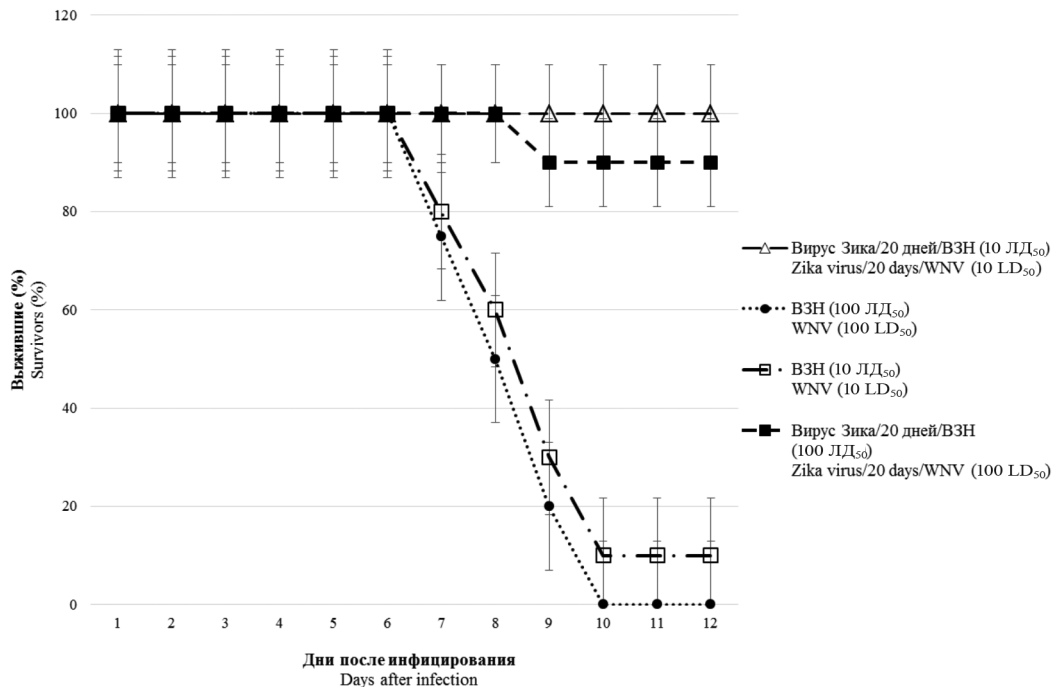


Fig. 2. Survival of Zika virus pre-infected BALB/c mice upon subsequent infection with lethal doses of West Nile virus (WNV).
△ – experimental results obtained by intraperitoneal infection with Zika virus (10^4 TCID₅₀) and after 20 days intraperitoneal infection with WNV (10 LD₅₀);
● – experimental results obtained by intraperitoneal infection with WNV (100 LD₅₀); □ – experimental results obtained with intraperitoneal infection of WNV (10 LD₅₀); ■ – experimental results obtained by intraperitoneal infection with Zika virus (10^4 TCID₅₀) and after 20 days intraperitoneal infection with WNV (100 LD₅₀) ($n = 10$; $p < 0.05$).

Рис. 2. Выживаемость предварительно инфицированных вирусом Зика мышей BALB/c при последующем инфицировании летальными дозами вируса Западного Нила (ВЗН).
△ – результаты, полученные при внутрибрюшинном инфицировании вирусом Зика (10^4 ТЦД₅₀) и через 20 сут при внутрибрюшинном инфицировании ВЗН (10 ЛД₅₀); ● – при внутрибрюшинном инфицировании ВЗН (100 ЛД₅₀); □ – при внутрибрюшинном инфицировании ВЗН (10 ЛД₅₀); ■ – при внутрибрюшинном инфицировании вирусом Зика (10^4 ТЦД₅₀) и через 20 сут при внутрибрюшинном инфицировании ВЗН (100 ЛД₅₀) ($n = 10$; $p < 0,05$).

Table 2. Induction of virus-neutralizing antibodies in BALB/c mice pre-infected with Zika virus and after 20 days infected with West Nile virus (WNV) (8 days after WNV infection)
Таблица 2. Индукция вируснейтрализующих антител у мышей BALB/c, инфицированных вирусом Зика и через 20 сут зараженных вирусом Западного Нила (ВЗН) (8 сут после заражения ВЗН)

Mouse serum Сыворотки мышей	Virus neutralizing antibody titers ($M \pm SEM$) Титры вируснейтрализующих антител ($M \pm SEM$)	
	Zika virus Вирус Зика	WNV ВЗН
Mono infection Моноинфекция		
Zika virus (20 days) Вирус Зика (20 сут)	82.3 ± 15.4	< 10
WNV (8 days)* ВЗН (8 сут)*	< 10	112.6 ± 27.2
Co-infection Коинфекция		
Zika virus/20 days/WNV (8 days) Вирус Зика/20 сут/ВЗН (8 сут)	74.5 ± 17.7	525.7 ± 73.5
Control Контроль	< 10	< 10

Note. * – death of 100% of animals to tenth day after infection with 100 LD₅₀ of WNV. The corresponding dilution of an individual animal serum neutralizing 50 TCID₅₀ of Zika virus or WNV in the neutralization test was taken as a titer. Geometric mean titers of virus neutralizing antibodies are presented ($n = 5$; $p < 0.05$).
Примечание. * – гибель 100% животных к 10-м суткам после инфицирования 100 ЛД₅₀ ВЗН. За титр принимали соответствующее разведение индивидуальной сыворотки животного нейтрализующей 50 ТЦД₅₀ вируса Зика или ВЗН в реакции нейтрализации. Представлены среднегеометрические титры вируснейтрализующих антител ($n = 5$; $p < 0,05$).

Conclusion

Thus, the study of mixed infection of two orthoflaviviruses in an *in vitro* model showed the presence of pronounced viral interference in the Vero E6 cell culture. Thus, WNV, when simultaneously and sequentially infected, inhibited Zika virus replication in cell culture by more than 1000 times, while the reverse effect did not exceed 30 (1.5 log10). Modeling mixed infection *in vivo* showed a more complex picture of the infectious process development in BALB/c mice. In simultaneous mixed infection of mice, the Zika virus did not significantly affect the development of lethal infection caused by WNV. At the same time, prior infection of mice with non-lethal doses of the Zika virus 4 and 20 days before the administration of WNV provided effective protection for the animals against lethal WNV infection and the emergence of a high level of virus-neutralizing antibodies against this virus.

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