

Influence of Protective Media Composition and Storage Temperatures on Preservation of Rabies Virus Vaccine Strain L. Pasteur

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Abstract: Here, we studied the effect of protective media supplemented with sucrose, glycerol, and maltose on the preservation of the rabies virus vaccine strain L. Pasteur during storage at various temperatures. The temperature of 37°C was found not to be suitable for virus storage. If needed in production the temperature of 5°C can be used for virus storage up to two weeks in protective medium with 5% glycerol, which allows preserving 81% from initial activity in these conditions. At –20 and –80°C, after 24 months of storage, a decrease in virus activity to non-valid level to be used in rabies products manufacturing was observed, which is likely due to the influence of “a solution effect” and “time recrystallization”. Considering the production need to use these temperature conditions for storage of large volumes of virus and according to the findings, it is allowed to store it at –20°C for up to six months in DMEM-based preserving medium containing 0.5% human albumin, with the addition mixture of 5% sucrose and 5% glycerol. Such conditions ensured the preservation of 80% from virus initial activity. Storage at –80°C is allowed in the medium with 5% sucrose for up to 12 months, which enables the preservation of 82% from virus activity. It has been shown that temperature of –196°C and protective media with addition of 5% sucrose, 5% glycerol and their mixture provide high infectious activity (83–87% from initial data) of rabies virus vaccine strain L. Pasteur after 24 months (observation period), which allows us to recommend these conditions for storage of reference samples and small volumes of virus.

Key words: rabies virus, cell culture, long-term storage, protective media, virus preservation, virus infectious activity.

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I. Introduction

Among other infectious diseases the rabies is a dangerous viral disease that affects the central nervous system and has the highest mortality rate. The main anti-rabies measures are vaccination of domestic and wild animals and pre- and post-exposure prophylaxis in humans using rabies vaccines and immunoglobulins [[1]–[8]]. Considering that the immunogenicity of these rabies products directly depends on the activity of virus used in their manufacturing, an important aspect is the effective storage of rabies virus vaccine strains [[1], [2], [6], [9]].

Freeze-drying and storage at low temperatures are recognized as the most common methods for viral specimens storage. Applying of virus freeze-drying under manufacturing conditions is limited by the need for additional seeding. Therefore, freezing at temperatures from –20 to –85°C in commercial chambers, allowing storage of large volumes of virus, is most widespread. To increase storage efficiency of viral material, di- and polysaccharides, alcohols, DMSO, animal blood serum and albumin, and other organic substances are used as protective additives for storage media [[2], [9]–[18]]. However, the features of low-temperature storage of rabies virus strains under manufacturing conditions have been little studied. In this regard, the urgent problem of the biotechnological industry is the development of individual preserving media for storage of specific virus strains at various low temperatures [[11], [12]].

JSC “BIOLIK” (Kharkiv, Ukraine) develops the technologies for rabies products manufacturing, which include storage of rabies virus vaccine strain L. Pasteur.

The research **aim** was to develop the preserving media compositions for storage of rabies virus vaccine strain L. Pasteur at various temperatures under manufacturing conditions of rabies vaccine for human use.

II. Materials And Methods

2.1 Virus and cells

The research object was rabies virus fixed vaccine strain L. Pasteur provided by Pasteur Institute (Novi Sad, Serbia) [11]. This strain was deposited (certificate No. 678) at Depository of the State Scientific Control Institute of Biotechnology and Strains of Microorganisms (Kyiv, Ukraine).

The permanent cell line Vero, obtained from the European Collection of Authenticated Cell Cultures (ECACC, UK), was used for virus amplification. This cell culture is recommended by the World Health Organization (WHO) and other international organizations as a substrate for virus propagation during manufacturing of rabies vaccines for human use [[1]–[3], [6], [8], [11], [19]].

2.2 Virus suspension preparation

Before infection, the Vero cell line was cultivated for 1 day in the near-wall monolayer in sterile plastic culture flasks (SPL, Germany) in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% (v/v) fetal bovine serum (Sigma-Aldrich, USA) in CO₂-incubator (Binder, Germany) at 37°C and 5% CO₂. The cell culture was infected by the virus with infectious activity (5.14 ± 0.14) Ig CCID₅₀ (50% cell culture infectious dose). With this aim the cells were exposed to virus suspension for an hour in CO₂-incubator at 33°C and 5% CO₂. Thereafter, virus DMEM-based growth medium (GM) supplemented with 0.5% (v/v) human albumin (Sigma-Aldrich, USA) was added to culture flasks and virus was cultivated the same as described above. On the 4th day after infection, the virus suspension was collected and purified from cell debris by centrifugation in refrigerated centrifuge (MPW, Poland) at 4°C (2000 g, 15 min) [[1]–[3], [7], [10], [15], [17]]. After supernatant collection, sucrose (AppliChem, Germany), glycerol (AppliChem, Germany) and maltose (Sigma-Aldrich, USA) were added to it in various concentrations (v/v). The following protective media were used to store the virus: 1 – GM; 2 – GM with 5% sucrose; 3 – GM with 5% glycerol; 4 – GM with 5% sucrose and 5% glycerol; 5 – GM with 5% maltose.

Virus suspension in specified protective media was poured using a variable volume mechanical dispenser (Biohit Proline Plus, Finland) into 1 ml sterile plastic cryovials (SPL, Germany) and placed for storage. To conduct an accelerated aging test, which is used in the pharmaceutical industry to assess the stability and shelf life of drugs, the virus was stored at 37°C in thermostat (Binder, Germany). In order to study the possibility of storing the viral suspension, excluding freezing and thawing, refrigerator (Liebherr, Germany) was used at 5°C. Also, the virus was placed in freezers (National Lab, Germany) for storage at –20 and –80°C, Dewar flask (Kharkov Plant of Transport Machinery, Ukraine) for storage at –196°C. The cooling rates to subzero temperatures were not controlled. The virus infectious activity was determined before storage in all investigated protective media. Preservation of samples stored at 37 and 5°C was evaluated after 5, 10, 15, 20, 30, 45, 60, 90 and 180 days (observation period). Infectious activity of virus stored at –20, –80, and –196°C was determined after a week, 3, 6, 12, 18, and 24 months of storage (observation period).

2.3 Virus infectious activity determination

Virus infectious activity was determined according to the WHO recommendations by titration in BHK-21 (clone 13) cell culture, which was obtained from the European Collection of Authenticated Cell Cultures. Titration was performed in 96-well culture plates (TPP, Switzerland) in 5 repetitions with dilution factor of 5. The cells in plates were cultivated in CO₂-incubator at 37°C and 5% CO₂ for 48 hours, fixed with acetone cooled at –20°C and stained with specific monoclonal antibodies to rabies virus labeled with fluorescein isothiocyanate (Fujirebio, USA). Using microscope (Leica DM2000, Germany) with fluorescence module at ×100 magnification, the presence of bright specific green glow in each well of plate, which indicated infection of cells with rabies virus, was taken into account. The value of virus infectious activity was calculated by the Spearman-Kärber method and expressed in Ig CCID₅₀ [[1], [2], [6], [7], [10], [15], [17]].

To assess the effectiveness of virus storage, the data on the infectious activity of samples in GM prior to manipulations were used as a control. When studying the dynamics of virus activity during storage at each temperature, the significance of changes in activity compared with the previous storage period was analysed. After 24 months of storage, the results were compared with virus infectious activity in medium 1 (GM without additives) at each storage temperature, and differences in the efficiency of temperature conditions were evaluated.

2.4 Statistical Analysis

The results were statistically analysed using Excel (Microsoft, USA) and Statistica 10 (StatSoft, USA) software as well as the analysis of variance method (ANOVA) was applied, i.e. the significance of differences between the means was determined and the influence significance of each factor on virus activity was evaluated. Differences were considered as significant at $p < 0.05$ [20].

III. Results And Discussion

3.1 Choice of control, contribution of different factors to changes in virus infectious activity

Due to the fact that addition of protective substances to GM before virus storage did not significantly affect its activity, the infectious activity in GM prior to manipulations (hereinafter, the initial virus infectious activity) was used as a control, which amounted to (5.45 ± 0.19) lg CCID₅₀. Taking into account the regulatory requirements for rabies vaccines manufacturing, the effectiveness of virus storage was confirmed while maintaining at least 80% of initial virus infectious activity.

It was found that during storage of the rabies virus strain L. Pasteur in different protective media at 37, 5, -20, -80 and -196°C, the changes of its infectious activity was influenced by protective medium composition, temperature and storage period. The storage temperature contributed greatly, whereas the storage period and protective medium composition affected less.

3.2 Infectious activity of rabies virus vaccine strain L. Pasteur during storage in different protective media at 37°C

After 5 days of storage at 37°C, a significant decrease in infectious activity of rabies virus vaccine strain L. Pasteur compared with the control occurred in all the studied protective media (Fig. 1). At the same time, in samples with GM without additives, 62% of the initial activity value was preserved, in samples with 5% glycerol, 5% sucrose, mixture of 5% sucrose and 5% glycerol, 5% maltose it was preserved 52, 40, 34, and 19% of it, respectively. After 10 and 15 days, further infectious activity decrease was observed in all the studied protective media. Virus was completely inactivated between 20 and 30 days of storage.

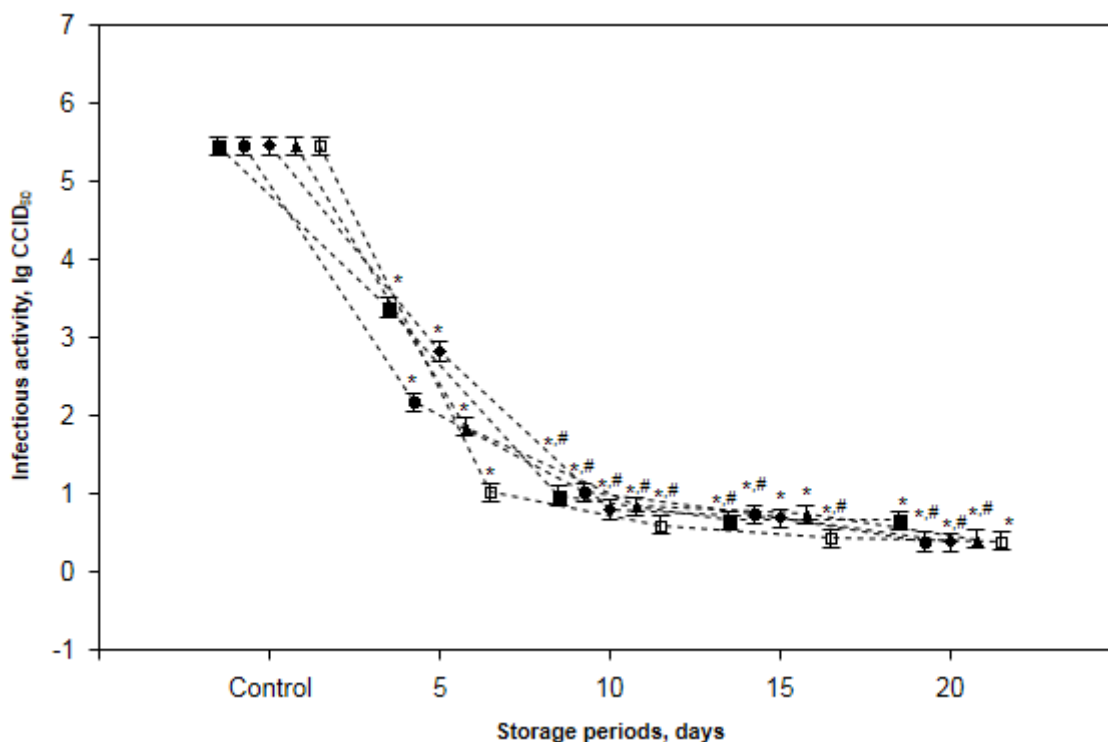


Fig 1: Infectious activity of rabies virus vaccine strain L. Pasteur during storage in different protective media at temperature of 37°C: 1 (■); 2 (●); 3 (◆); 4 (▲); 5 (□); * – differences are significant as compared with control; # – differences are significant as compared with previous storage period (p < 0.05; n = 5).

3.3 Infectious activity of rabies virus vaccine strain L. Pasteur during storage in different protective media at 5°C

During storage at 5°C for 5 days, a significant decrease in the infectious activity of the vaccine strain L. Pasteur compared with the control occurred in all the studied protective media (Fig. 2). The highest preservation indices for this period were found in the samples with 5% glycerol (91% of the initial activity), without additives (87%) and with 5% sucrose (82%). In other media, less than 80% of the initial value was preserved. Within 15 days of storage, only the medium with 5% glycerol ensured the virus preservation that met regulatory requirements, which after 10 and 15 days amounted to 86 and 81% of the control value, respectively. Further storage led to a decrease in virus activity in all studied protective media. After 6 months (180 days) of

storage, the highest virus preservation under these conditions was noted in GM without additives (38% of the initial infectious activity). Between 6 and 12 months, the virus was completely inactivated in all samples.

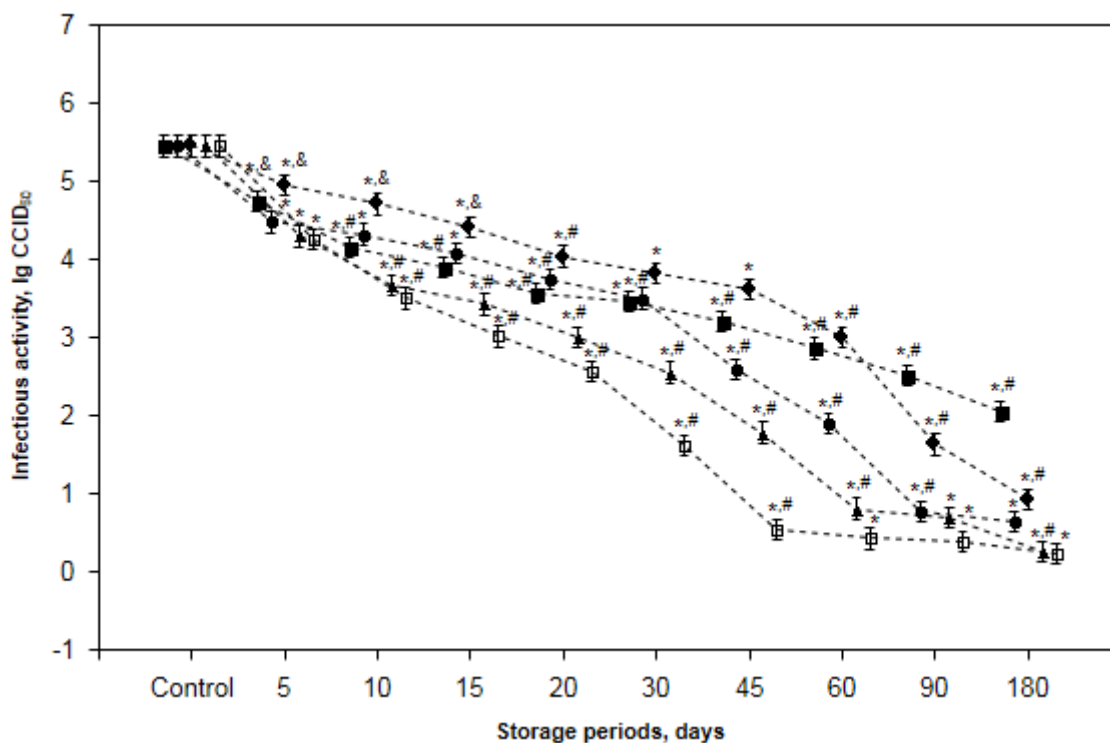


Fig 2: Infectious activity of rabies virus vaccine strain L. Pasteur during storage in different protective media at temperature of 5°C: 1 (■); 2 (●); 3 (◆); 4 (▲); 5 (□); * – differences are significant as compared with control; # – differences are significant as compared with previous storage period; & – virus preservation is higher than 80% and higher than in other studied protective media at the same storage period ($p < 0.05$; $n = 5$).

3.3 Infectious activity of rabies virus vaccine strain L. Pasteur during storage in different protective media at -20, -80 and -196°C

The dynamics of changes in the vaccine strain L. Pasteur infectious activity at -20, -80 and -196°C was evaluated within 24 months (observation period) separately in each of the protective media. One week after being stored in GM without additives (medium 1), the virus activity was significantly lower than the control at all temperatures (Fig. 3). At this storage period at -20°C, the studied parameter decreased to 85% of the initial value and remained at this level for up to 3 months. Between 3 and 18 months there was a decrease in the virus infectious activity, after which it was stable until 24 months of storage and amounted to 53% of the control value (Fig. 4). The similar dynamics in the virus activity was observed at -80°C. After 24 months of storage under these conditions, 67% of the initial value was preserved. The temperature of -196°C, like others, caused a partial death of the viral particles during the freezing stage, which led to the loss of 21% activity. During further storage for up to 24 months at -196°C, the virus infectious activity did not change.

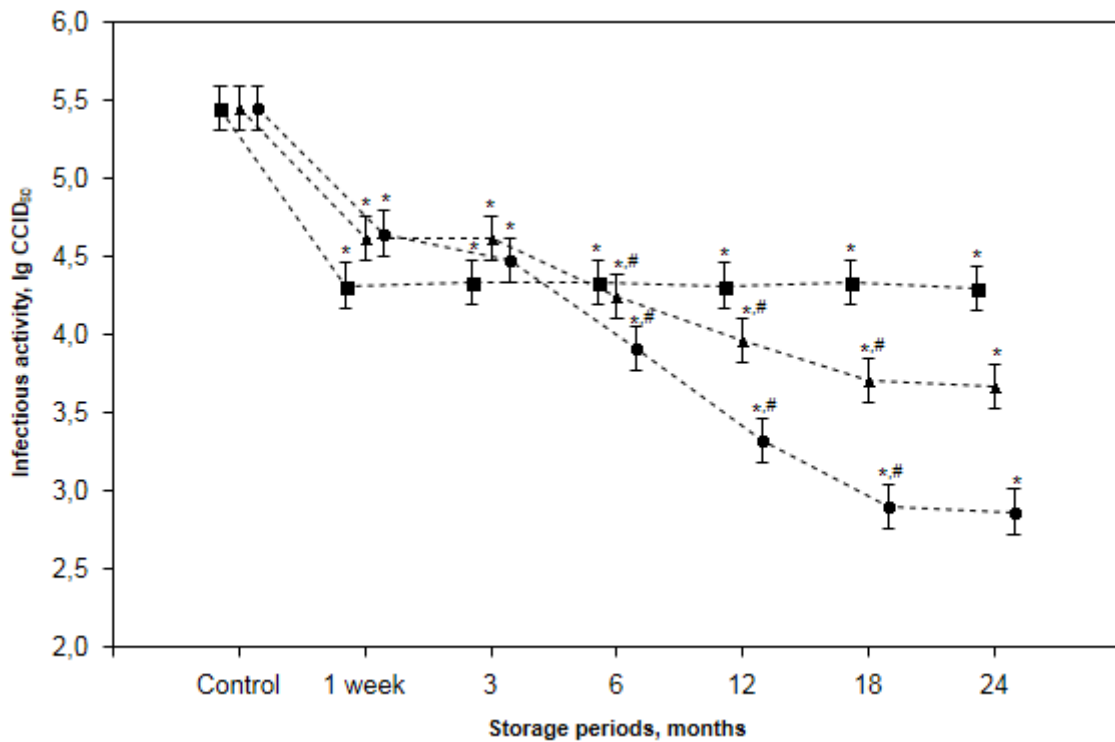


Fig 3: Infectious activity of rabies virus vaccine strain L. Pasteur during storage in GM without additives at different temperatures: -20 (●); -80 (▲); -196°C (■); * – differences are significant as compared with control; # – differences are significant as compared with previous storage period (p < 0.05; n = 5).

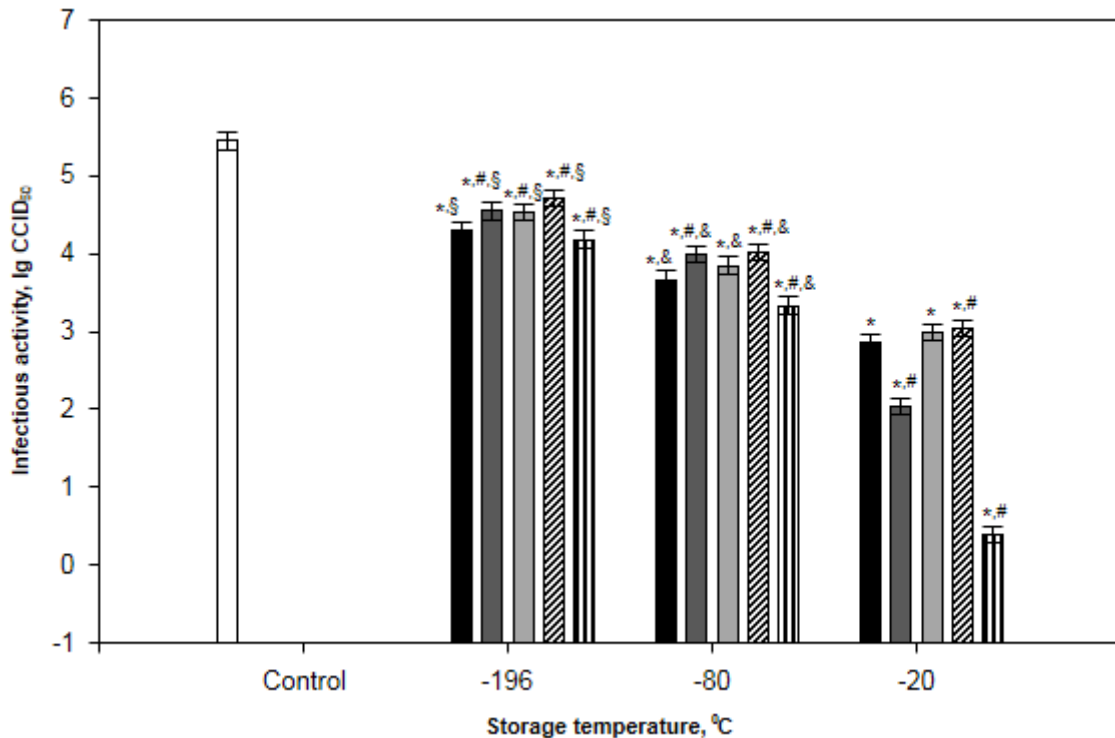


Fig 4: Infectious activity of rabies virus vaccine strain L. Pasteur after 24 months of storage in different protective media: control (□); 1 (■); 2 (▒); 3 (▓); 4 (▨); 5 (▩); * – differences are significant as compared with control; # – differences are significant as compared to medium 1 at each temperature; & – infectious activity is significantly higher than at -20°C; § - infectious activity is significantly higher than at -20 and -80°C (p < 0.05; n = 5).

In protective medium supplemented with 5% sucrose (medium 2), after a week of storage at -20°C , the virus infectious activity significantly decreased as compared to the control and amounted to 86% of its value, and later it remained stable up to 3 months (Fig. 5). With further storage of the virus, the studied index decreased and after 24 months amounted to 37% of the control value (Fig. 4). At -80°C , the dynamics of activity of the samples with sucrose was similar to that in GM without additives at this temperature, and after 24 months 73% of the initial infectious activity remained in them. During the week of storage at -196°C the virus activity did not change. After 3 months, it decreased by 9% and remained stable until 18 months, and then it continued to decline and after 24 months amounted to 83% of the initial value.

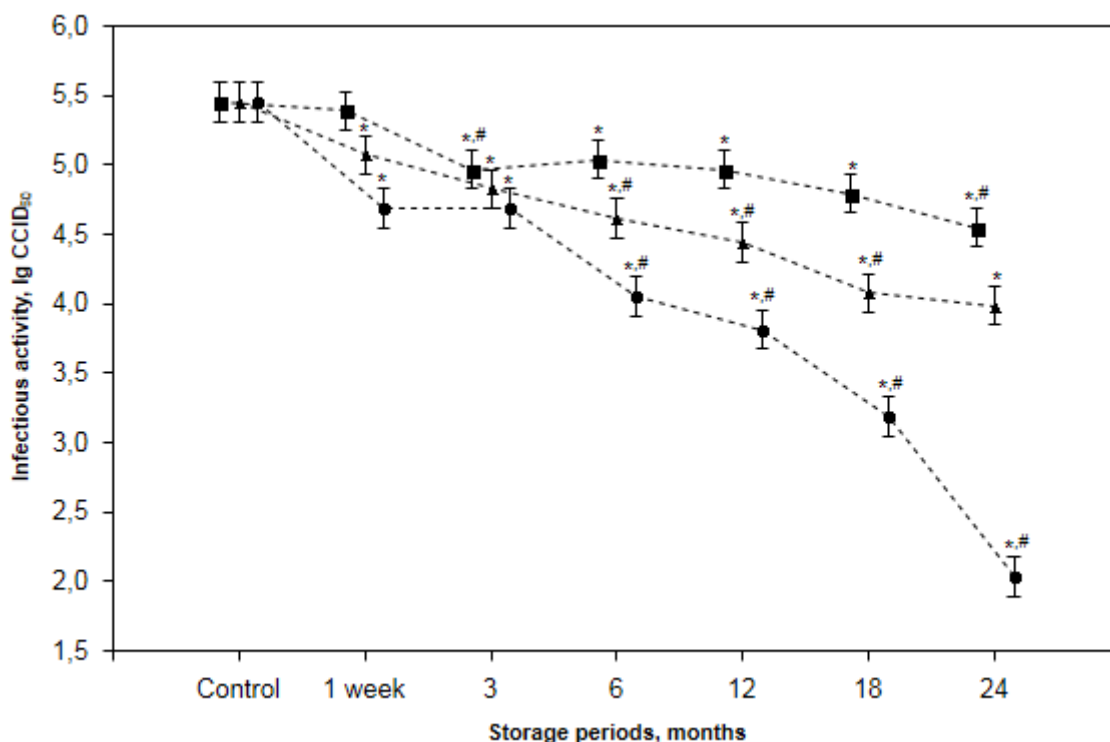


Fig 5: Infectious activity of rabies virus vaccine strain L. Pasteur during storage in GM with 5% sucrose at different temperatures: -20°C (●); -80°C (▲); -196°C (■);* – differences are significant as compared with control; # – differences are significant as compared with previous storage period ($p < 0.05$; $n = 5$).

During the weekly storage of virus in GM with addition of 5% glycerol (medium 3), its activity was significantly lower than the control at all temperatures (Fig. 6). At -20°C the virus preservation rate decreased between 3 and 6 and between 12 and 24 months, and at the end of the observation period amounted to 55% of the initial value (Fig. 4). During storage at -80°C , the virus infectious activity decreased between 6 and 18 months and after 24 months amounted to 71% of initial value. The temperature regimen of -196°C ensured the virus stability between 1 week and 18 months of storage. After 24 months at this temperature, 83% of the control activity was kept.

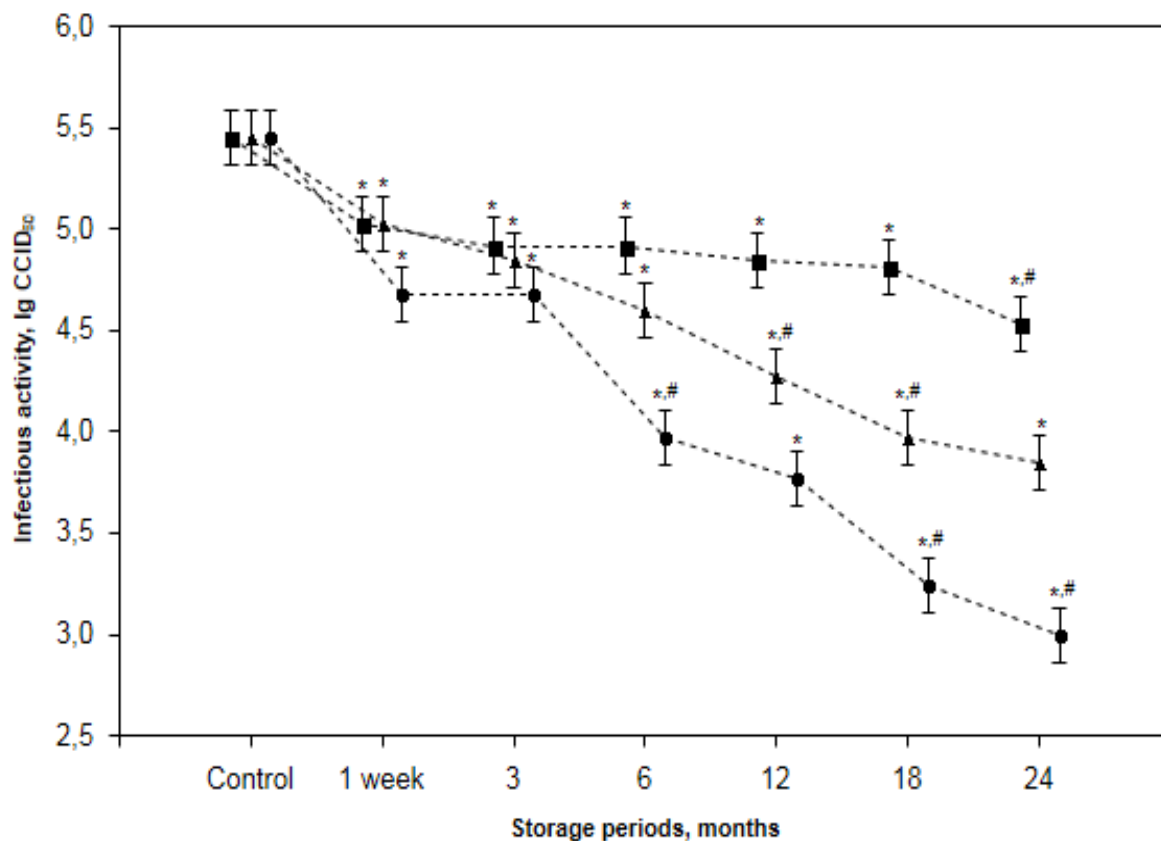


Fig 6: Infectious activity of rabies virus vaccine strain L. Pasteur during storage in GM with 5% glycerol at different temperatures: -20 (●); -80 (▲); -196°C (■);* – differences are significant as compared with control; # – differences are significant as compared with previous storage period (p < 0.05; n = 5).

In GM supplemented with 5% sucrose and 5% glycerol (medium 4), the virus infectious activity at -20°C decreased during the week and after 3 months of storage until the end of the observation period (Fig. 7). After 24 months, 56% of its initial value was preserved (Fig. 4). At -80°C, after the week of storage in this medium, the virus activity decreased by 13% compared with the control and remained stable up to 3 months. Between 3 and 12 months, a decrease in the samples preservation rate was observed, after which it remained at the previous level until 24 months of storage and amounted to 74% of the control activity. The temperature of -196°C ensured the stability of the virus during its freezing and storage for a week. After 3 months, the virus activity under these conditions decreased by 10% and remained at the same level until 18 months, then it began to decline and after 24 months it amounted to 87% of the initial value.

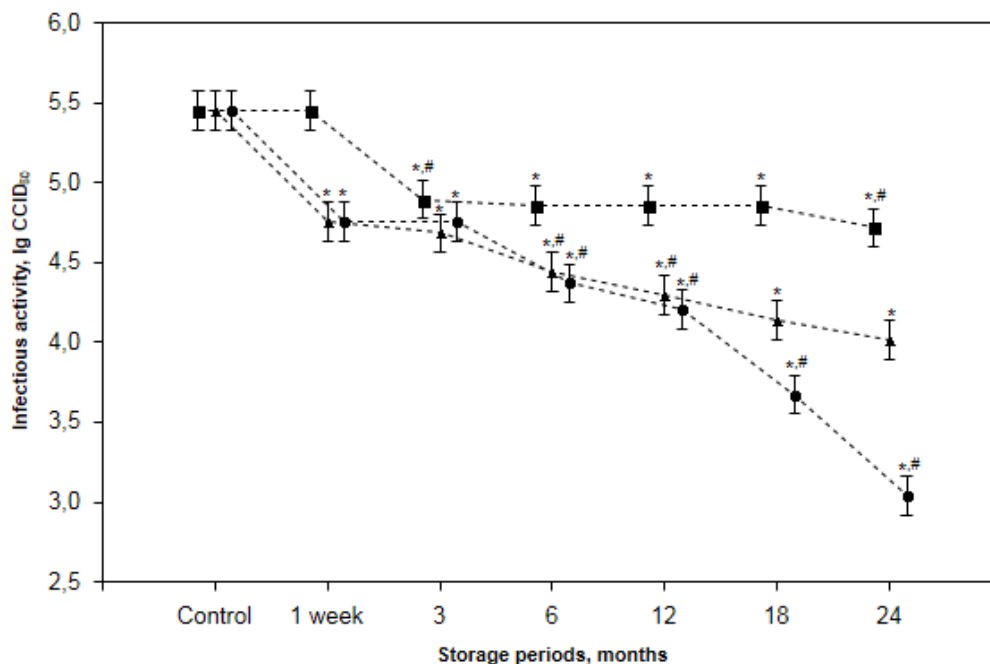


Fig 7: Infectious activity of rabies virus vaccine strain L. Pasteur during storage in GM with 5% sucrose and 5% glycerol at different temperatures: -20°C (●); -80°C (▲); -196°C (■); * – differences are significant as compared with control; # – differences are significant as compared with previous storage period ($p < 0.05$; $n = 5$).

At -20°C in protective medium with the addition of 5% maltose (medium 5), a decrease in the virus infectious activity occurred throughout all 24 months of storage (Fig. 8). After the end of the observation period, under these conditions, 7% of the initial activity was preserved (Fig. 4). At -80°C , the virus activity decreased within 3 months, between 6 and 12, as well as 18 and 24 months of storage, amounting to 61% of the control activity. In the medium with maltose the temperature of -196°C provided preservation of viral particles in the stages of freezing and storage for a week. During further storage for 3 months, the virus activity decreased, but remained stable until 24 months, amounting to 77% of the initial rate.

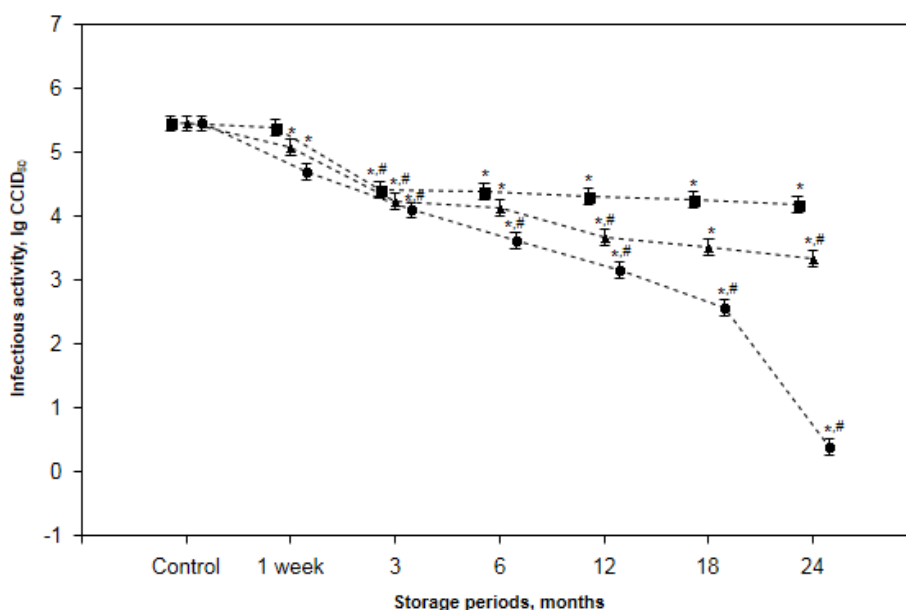


Fig 8: Infectious activity of rabies virus vaccine strain L. Pasteur during storage in GM with 5% maltose at different temperatures: -20°C (●); -80°C (▲); -196°C (■); * – differences are significant as compared with control; # – differences are significant as compared with previous storage period ($p < 0.05$; $n = 5$).

When studying the effect of protective substances added to GM on the virus preservation, the following results were obtained. After 24 months of storage, the virus infectious activity was higher as compared to the medium without additives (medium 1) in the medium with mixture of sucrose and glycerol at the temperature of -20°C (by 3%); sucrose (by 6%) and mixture of sucrose and glycerol (by 7%) at -80°C ; sucrose (by 4%), glycerol (by 4%) and their mixture (by 8%) at -196°C (Fig. 4). In other media, the preservation rate did not differ or was lower than in medium 1. In all the studied protective media, the activity of the virus stored at -196°C was higher than at other temperatures, at -80°C it was higher than at -20°C .

3.4 Analysis of results

The obtained results indicate the unsuitability of the temperature of 37°C for virus storage. At 5°C it can be stored for up to 15 days in GM with addition of 5% glycerol.

The experiment results show that differences in the structure of viruses and cells do not affect the response of rabies virus to freezing and storage temperature conditions, which corresponds to the basic principles of generally accepted concepts of cell cryodamage and cryoprotection, in particular, two-factor and multifactor theories of cryoinjuries [[16], [21]]. According to these theories, the preservation of biological specimens during low-temperature preservation largely depends on the speed and mode of cooling, the presence of cryoprotective substances in the preserving medium and storage temperature.

Taking into account that the temperature range from -10 to -80°C refers to the crystallization zone of cooled and overcooled water [[17]], and, considering the eutectic temperatures for solutions of various electrolytes and cryoprotectants [[18]], it can be assumed that the virions stored at -20°C in multicomponent protective media were constantly in a liquid phase in microchannels between ice crystals. This led to the combination effect of damaging factors: hyperconcentration of salts and other components, changes of the medium pH, dehydration of macromolecules, and disturbances in intermolecular interactions (“a solution effect”) [[3], [22]]. In addition, under the low temperatures influence, loosening and aggregation of nucleocapsid proteins [18] and damage to the viral RNA-dependent RNA polymerase can occur. Virus samples stored at -80°C were affected by the same physicochemical factors, however, less pronounced, because after 24 months, preservation indices at a given temperature were higher than at -20°C . With increasing length of storage at this temperature, “time recrystallization” occurred, which led to a further ice crystals enlargement with an increase in the salts and other components concentration, consequently, an intensification of the “a solution effect”, which, presumably, caused a decrease in the virus infectious activity between 3 and 24 months of storage. The dynamics of decrease in the virus activity at -20 and -80°C may indicate that in addition to the virions death as a result of a direct effect of low temperature and “a solution effect”, non-lethal and sub-lethal cryoinjuries could accumulate in the virions during storage. These injuries were realized upon additional exposure of damaging physicochemical factors at the stage of samples heating.

Higher virus preservation rates in cryoprotectants-containing media as compared to medium without additives indicate that these substances reduced the eutectic point in stored samples. The shown protective effect from adding penetrating (glycerol) and non-penetrating (sucrose) cryoprotectants into the GM indicates the manifestation of their hydration properties during freezing and low-temperature storage of the samples. This is supported by higher virus preservation in medium with sucrose as compared with maltose, which has a lower ability to bind water [[18]]. In addition, the results of the virus preservation in GM without cryoprotectants indicate that the studied DMEM-based protective media, supplemented with human albumin seem to stabilize the capsid and supercapsid of virions.

IV. Conclusions

1. It was established that the storage temperature of -196°C and protective media based on the virus GM (DMEM with 0.5% human albumin) provided high preservation rates for the infectious activity of the rabies virus vaccine strain L. Pasteur for 24 months (observation period). The highest virus preservation under these conditions was ensured by protective media based on GM with addition of 5% sucrose (83% of the initial control), 5% glycerol (83%), and their mixtures (87%).
2. The temperature mode of -80°C and the medium supplemented with 5% sucrose provided high virus preservation (82% of the initial indicator) up to 12 months of storage.
3. Cryoprotective effect of media with the addition of sucrose and glycerol was shown to be due to their hydration properties and stabilization of virions.
4. Considering the regulatory requirements for the rabies vaccines manufacturing, for long-term storage of large volumes of the rabies virus vaccine strain L. Pasteur, the temperature of -80°C and protective medium based on GM with the addition of 5% sucrose were recommended. For storage of reference samples and small virus volumes, the temperature of -196°C and protective media based on GM with the addition of 5% sucrose, 5% glycerol and mixture of 5% sucrose and 5% glycerol are recommended.

5. In case of production need, it is allowed to store the virus at -20°C in protective medium based on GM with addition of 5% sucrose and 5% glycerol (up to 6 months); at 5°C in medium with addition of 5% glycerol (up to two weeks).

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