IOSR Journal Of Pharmacy
(e)-ISSN: 2250-3013, (p)-ISSN: 2319-4219
Volume 9, Issue 10 Series. I (October 2019), PP. 09-18
www.iosrphr.org

Protective Media for Storage of L. Pasteur Rabies Virus Strain at Different Temperatures

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Received 16 October 2019; Accepted 31 October 2019

Abstract: Here, we studied various preserving media compositions for long-term storage of L. Pasteur rabies virus vaccine strain at –20 and –80ºC in rabies products pharmaceutical manufacturing. Differences in strain infectious activity were established after storage at –80 and –20ºC. Infectious activity of the virus stored at –80ºC was higher than in the samples stored at –20ºC, the differences were statistically significant. During storage at these temperatures within a year (observation period), the highest safety of L. Pasteur rabies virus strain was provided with preserving media based on growth medium without additives and supplemented with sucrose, gelatin, glycerol and DMSO. Considering regulatory requirements and rabies products manufacturing conditions, protective media based on DMEM with 0.1% BSA, both without cryoprotectants and with the addition of 2.5-10% sucrose and storage at –80ºC were recommended for the long-term preservation of the studied rabies virus strain.
***Key words*:** cell culture, long-term storage, protective media, rabies virus, virus infectious activity, virus safety.

I. INTRODUCTION

Rabies is a zoonotic viral neuroinfection, characterized by severe encephalitis development. Without specific immunotherapy, this disease is fatal. Vaccines are used for rabies pre-exposure prophylaxis, vaccines and specific immunoglobulins are applied for post-exposure prophylaxis [[1]–[3]]. The important element of the rabies products manufacturing is the virus strains banks system, ensuring high safety of their infectious activity and, therefore, vaccines immunogenicity [[3]–[6]].

Freeze-drying and storage at low temperatures are the most effective methods for viruses long-term storage in the microorganisms collections and in pharmaceutical industry [[1], [2], [4], [6]–[13]]. Lyophilization with periodic passaging is now more often used for vaccine strains storage in mass rabies products manufacturing. At the same time, low-temperature storage is more efficient and profitable at all the manufacturing stages. However, features of such a storage method in rabies products manufacturing have been poorly studied.

For RNA-containing viruses storage at low temperatures, preserving media containing various cryoprotective and stabilizing capsid of virions substances are used: oligosaccharides (sucrose, trehalose), polyhydric alcohols (glycerol, sorbitol), oxides (DMSO), salts (monosodium glutamate), plant and animal origin substances (sodium alginate, gelatin, peptone, cattle and other animals blood serum or its components) [[1], [4], [6]–12, [14]–[18]]. The vital biotechnological industry problem is individual preserving media development for specific virus strains storage at different low temperatures that meet production conditions [[8], [9], [11]].

In PJSC "Pharmstandard-Biolik" (Kharkiv, Ukraine), the rabies products industrial technologies using L. Pasteur rabies virus fixed strain are being developed. These technologies include the stages of virus long-term storage.

The research **aim** was to develop a preserving media composition for a long-term storage of L. Pasteur rabies virus fixed strain at –20 and –80ºC in rabies products pharmaceutical manufacturing.

II. MATERIALS AND METHODS

2.1 Virus and cells

The research objects was L. Pasteur vaccine strain, provided by Pasteur Institute (Novi Sad, Serbia). This strain was deposited (certificates No. 678) at Depositary of the State Scientific Control Institute of Biotechnology and Strains of Microorganisms (Kyiv, Ukraine).

The permanent BHK-21 (clone 13) cell line, obtained from the European Collection of Authenticated Cell Cultures (ECACC, UK), was used for virus propagation. This cell culture is recommended by the World

Health Organization, International Epizootic Bureau and other international organizations as a substrate for rabies virus propagation during rabies preparations production [[2], [3], [5]].

2.2 Preparation of virus suspensions

The BHK-21 cells were cultivated for 1 day in the near-wall monolayer in sterile plastic culture flasks (SPL, Germany) in a growth medium based on DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% (v / v) fetal bovine serum (Sigma-Aldrich, USA) in CO_2-incubator (Binder, Germany) at 37ºC and 5% CO_2. For cell culture infection, L. Pasteur strain with infectious activity (6.78 ± 0.15) lg $CCID_{50}$ (50% cell culture infectious dose) was used. The cells were exposed with virus suspension for 1 hour in CO_2-incubator at 33ºC and 5% CO_2. Thereafter, growth medium on the basis of DMEM supplemented with 0.1% (v / v) bovine serum albumin (BSA) was added to culture flasks and the virus was cultivated the same as described above. Virus suspension was harvested to day 4 after infection and purified from cellular debris by centrifugation in a refrigerated centrifuge (MPW, Poland) at 4ºC (2000 g, 15 min) [[1], [2], [3], [5], [13]]. After supernatant collection, the following protective substances were added to it: sucrose (AppliChem, Germany), glycerol (AppliChem, Germany), DMSO (AppliChem, Germany), gelatin (Genesis, Ukraine), sodium alginate (Sigma-Aldrich, Germany) and peptone (HiMedia Laboratories Pvt. Limited, India) in various concentrations (v/v) (Table 1).

Table 1: Protective media compositions used for storage of L. Pasteur rabies virus strain, concentrations (v/v)

Protective medium	Composition	Protective medium	Composition
1	growth medium (GM)*	4/3	GM with 7.5% DMSO
2/1	GM with 2.5% sucrose	4/4	GM with 10% DMSO
2/2	GM with 5% sucrose	5/1	GM with 1% gelatin
2/3	GM with 7.5% sucrose	5/2	GM with 3% gelatin
2/4	GM with 10% sucrose	6/1	GM % sodium alginate
3/1	GM with 2.5% glycerol	6/2	GM with 2% sodium alginate
3/2	GM with 5% glycerol	6/3	GM with 3% sodium alginate
3/3	GM with 7.5% glycerol	7/1	GM with 2.5% peptone
3/4	GM with 10% glycerol	7/2	GM with 5% peptone
4/1	GM with 2.5% DMSO	7/3	GM with 7.5% peptone
4/2	GM with 5% DMSO	7/4	GM with 10% peptone

* DMEM with 0.1% BSA

Virus suspension in specified protective media was poured using a mechanical dispenser with variable volume (Biohit Proline Plus, Finland) into 1 ml sterile plastic cryovials (SPL, Germany) and placed into freezers (National Lab, Germany) for storage at –20 and –80ºC. Samples safety was evaluated by determining the infectious activity before storage, within a week and after 1, 3, 6 and 12 months of storage (observation period).

2.3 Determination of virus infectious activity

Titration method in BHK-21 cells was used to determine virus infectious activity. Titration was performed in 96-well culture plates (TPP, Switzerland) in 5 repetitions with dilution factor of 5. The cells in plates were cultured in CO_2-incubator at 37ºC and 5% CO_2 for 48 hours and fixed with acetone cooled at –20ºC. To determine the virus activity, a fluorescence method was used. To do this, the cell monolayer was stained with specific monoclonal antibodies to rabies virus, labeled with fluorescein isothiocyanate (FITC) (Fujirebio, USA). The presence or absence of specific bright green luminescence in each well was taken into account using laboratory microscope (Leica DM2000, Germany) with a fluorescence module at ×100 magnification (Fig. 1A-F). The luminescence presence indicated that the cells were infected with rabies virus.

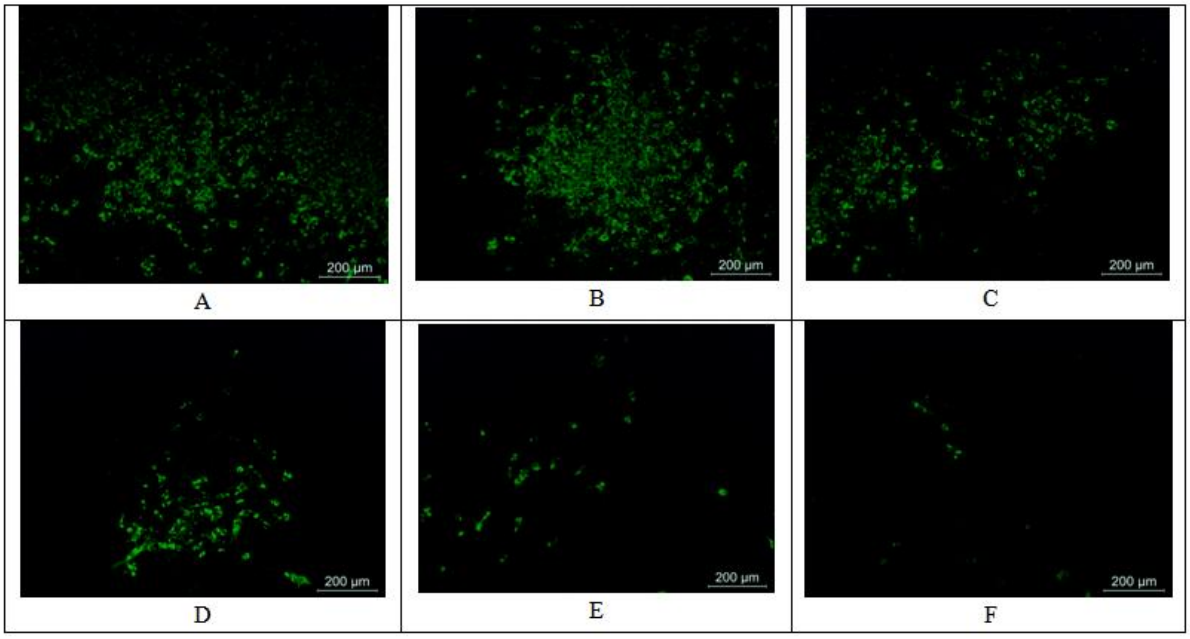

Fig 1: Monolayer of BHK-21 cells, infected with L. Pasteur rabies virus strain in various dilutions: (A) – 1:125, (B) – 1:625, (C) – 1:3125, (D) – 1:15625, (E) – 1:78125, (F) – 1:390625. Staining with specific monoclonal antibodies to rabies virus, labeled with fluorescein isothiocyanate, ×100 magnification.

The virus infectious activity was calculated using Spearman-Karber' method and expressed in decimal logarithm of 50% cell culture infectious dose (lg $CCID_{50}$):

$$\lg CCID_{50} = (x_0 - d/2 + d\Sigma r_i/n_i) \quad (1)$$

where x_0 is lg of inverse highest dilution, where the luminescence is observed in all the wells; d is lg of dilution factor; r_i is number of the wells with luminescence in each dilution of virus suspension titration; n_i is total number of the wells per each dilution of virus suspension titration [[1], [2], [13]].

To assess the effectiveness of preserving media during the storage of the virus, the data on the infectious activity of samples in the growth medium prior to manipulations were used as a control. Also, differences in the efficiency of temperature regimens –20 and –80ºC were evaluated. In addition, after 12 months of storage, the results were compared with the infectious activity of the virus in medium 1 (growth medium without additives) at each storage temperature. When assessing the dynamics of changes in virus activity during storage, the significance of changes in virus activity compared with the previous storage period was analyzed.

2.4 Statistical Analysis

The data were statistically processed with Excel (Microsoft, USA) and Statistica 10 (StatSoft, USA) software standard packages and analysis of variance method (one-way, factorial and main effects ANOVA), i.e. the significance of differences between the mean values was determined and the influence significance of each factor on activity variability was evaluated. Differences were considered as statistically significant at $p < 0.05$ [[19]].

III. RESULTS AND DISCUSSION

3.1 Contribution of different factors

During the study of the safety of L. Pasteur rabies virus strain samples after storage in various protective media at –20 and –80ºC for 12 months (observation period), the significance of each factor contribution (temperature, storage period and protective medium) was evaluated. It was established that during virus storage, all factors in varying degrees influenced the changes in specimens infectious activity according to descending order, the temperature affected greater, and then storage period and protective medium contributed less.

3.2 Infectious activity of L. Pasteur rabies virus strain after one week and 12 months storage in various protective media

It was found that the addition of the abovementioned protective substances to the growth medium did not significantly affect the virus infectious activity prior to manipulations. In this regard, the virus infectious

activity in the growth medium prior to manipulations (hereinafter, the initial virus infectious activity) was used as a control, which amounted to (7.23 ± 0.10) lg $CCID_{50}$.

After freezing and storage for a week at –20 and –80ºC, a significant decrease in its infectious activity was found in all the studied protective media, excluding the samples frozen at –80ºC in 6/1 and 6/2 media (growth medium supplemented with 1 and 2% sodium alginate) (Fig. 2). The virus safety after freezing at –80ºC was higher than at –20ºC, the differences were statistically significant.

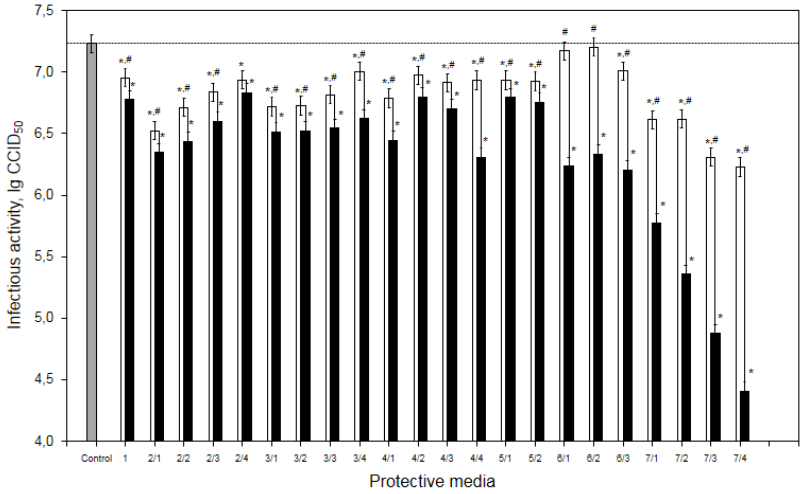

Fig 2: Infectious activity of L. Pasteur rabies virus strain after freezing in various protective media and one week storage; □ – at –80ºC; ■ – at –20ºC; ■ – control; * – differences are statistically significant as compared with control, # – infectious activity after freezing at –80ºC significantly higher than at –20ºC ($p < 0.05$; $n = 5$).

During storage at –20ºC after 1 month, an infectious activity in specimens with 10% glycerol (3/4) and gelatin (5/1 and 5/2) was higher than in medium 1 (growth medium without additives). In remaining samples, the virus preservation indices were lower than in medium without additives. After 3 months, a higher safety as compared with the medium 1 provided the samples with 7.5 and 10% sucrose (2/3 and 2/4), 5–10% glycerol (3/2–3/4) and gelatin (5/1 and 5/2), after 6 months, the samples with sucrose, glycerol, DMSO and gelatin in all concentrations under the test showed similar efficacy. After 12 months, the infectious activity of the virus stored in media with sucrose and in all the studied concentrations (2/1–2/4, 3/1–3/4), and DMSO at 5%concentration (4/2) was higher, than in a growth medium without additives (Fig. 3).

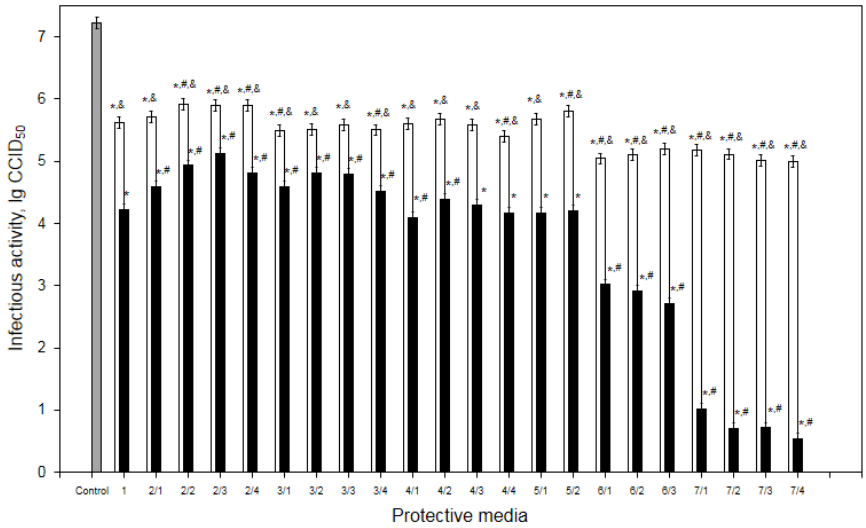

Fig 3: Infectious activity of L. Pasteur rabies virus strain after 12 months storage in various protective media; □ – at –80ºC; ■ – at –20ºC; ■ – control; * – differences are statistically significant as compared with control, # – differences are statistically significant as compared with medium 1; & – infectious activity after storage at –80ºC significantly higher than at –20ºC ($p < 0.05$; $n = 5$).

However, at –80ºC after 1 month of storage, in all the studied media, the safety indices were lower than in medium 1. After 3 months, significantly higher safety compared to the growth medium without additives was provided by the specimens with 5% DMSO, 3% gelatin and 2% sodium alginate (4/2, 5/2, 6/2); after 6 months it was provided by the samples with 7.5 and 10% sucrose (2/3 and 2/4), glycerol, DMSO, gelatin and sodium alginate in all concentrations under the test. After 12 months, the virus was more active in the media with 5–10% sucrose and 3% gelatin (2/2–2/4 and 5/2) than in the medium 1. During all storage periods, virus infectious activity at –80ºC exceeded the activity of virus stored at –20ºC. After 12 months of storage at –20ºC, the following virus safety results were obtained compared to the initial virus infectious activity. The growth medium with the addition of peptone ensured the safety of 7-14% of the initial virus infectious activity, the medium with sodium alginate allowed to save 38–42% of it, the medium without additives and with the addition of gelatin preserved 58% of initial activity, in the medium with DMSO there was preserved 57–61% of it, the media with glycerol and sucrose ensured the safety of 63–67 % and 64–71% of initial virus infectious activity respectively. At –80ºC, after 12 months of storage, in the medium with peptone, 69–71% of the initial virus activity was preserved, the medium with sodium alginate allowed to save 70–72% of it, in the medium with glycerol there was preserved 76–77% of the initial activity, in the medium without additives remained 78% of it, the medium with DMSO provided the safety of 75–79% of the initial activity, the media with gelatin and sucrose ensured the safety of 79–80% and 79–82% of initial virus infectious activity respectively.

3.3 Dynamics of changes in infectious activity of L. Pasteur rabies virus strain during storage at temperatures of –20 and –80ºC

To analyze the dynamics of changes in infectious activity of L. Pasteur rabies virus strain the preserving media with highest survival rates after 12 months were selected, namely growth medium without additives and with sucrose, glycerol, DMSO and gelatin. At the same time, the significance of differences in the infectious activity of the virus compared with the previous storage period in the interval between 1 week and 12 months was evaluated.

During the storage of virus in a growth medium without additives at –20ºC, a statistically significant decrease in its activity occurred at all storage periods (Fig. 4). At –80ºC, the virus activity was stable up to 1 month of storage, but decreased during its further storage.

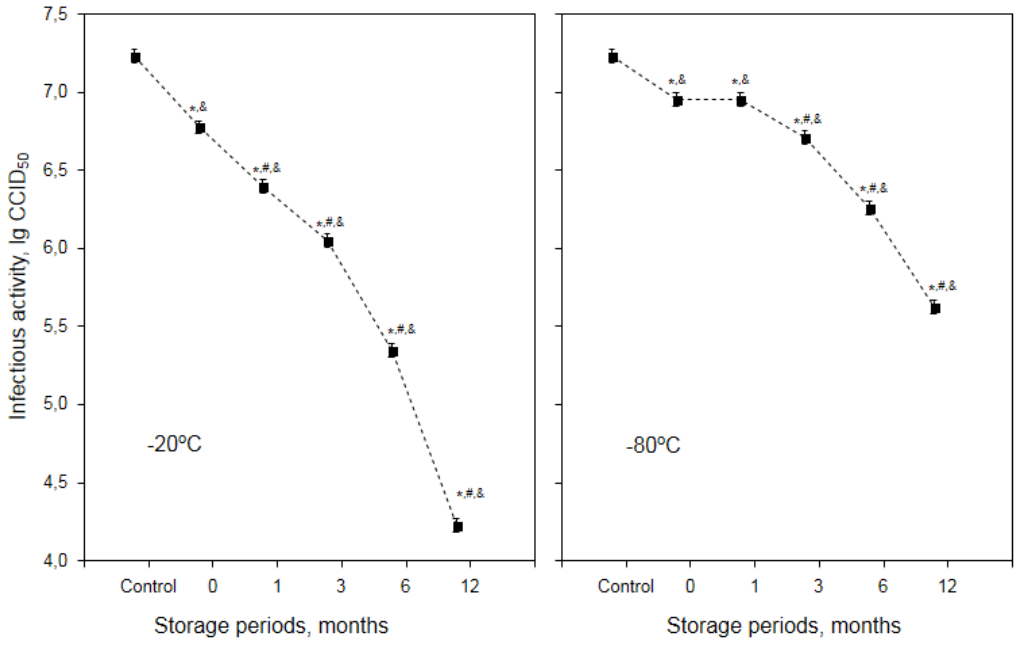

Fig 4: Dynamics of changes in infectious activity of L. Pasteur rabies virus strain during storage at temperatures of –20 and –80ºC in growth medium without additives; * – differences are statistically significant as compared with control, # – differences are statistically significant as compared with previous storage period; & – differences between infectious activity after storage at –20 and –80ºC are statistically significant ($p < 0.05$; $n = 5$).

During storage of the virus at –20ºC in media with sucrose a statistically significant decrease in its infectious activity occurred within the first three months (Fig. 5). Between 3 and 6 months, virus infectious activity, excluding the samples with 2.5% sucrose, remained stable. While in preparations with 2.5% sucrose partial inactivation of the virus occurred. There was a further decrease in activity between 6 and 12 months of storage in all specimens with sucrose. The highest virus safety after 12 months, 71% of the initial activity, was ensured by the medium with 7.5% sucrose. During virus storage at –80ºC in media with sucrose, a significant decrease in infectious activity occurred between 1-3 and 6-12 storage months. The highest virus safety after 12 months, 82% of the initial activity, was ensured by media with 2.5 and 5% sucrose.

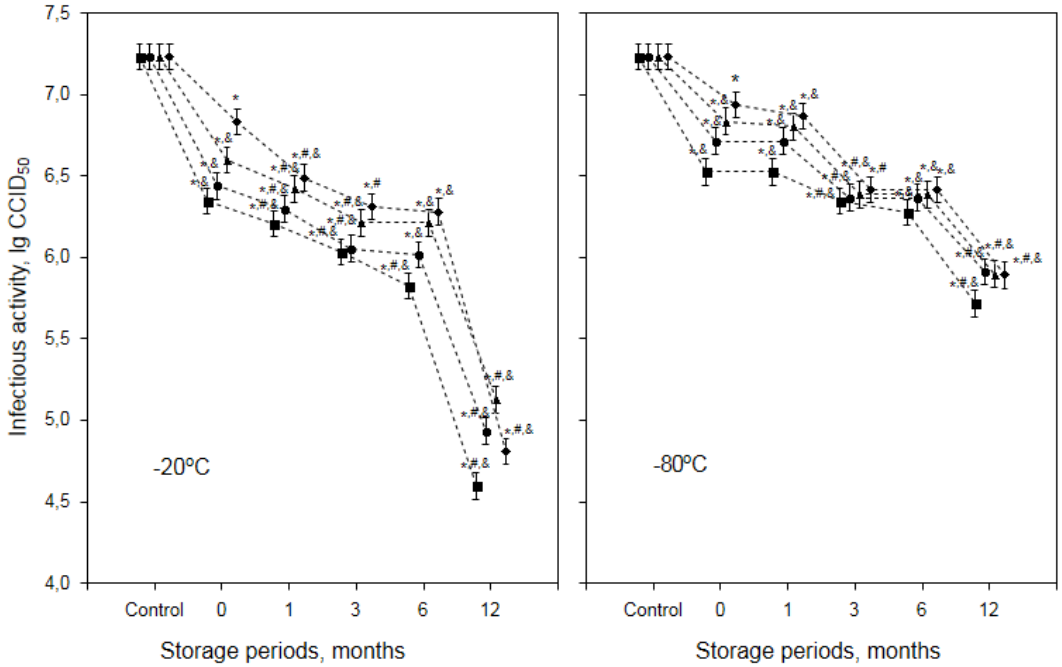

Fig 5: Dynamics of changes in infectious activity of L. Pasteur rabies virus strain during storage at temperatures of –20 and –80ºC in protective media with sucrose; ■ – 2.5%; ● – 5%; ▲ – 7.5%; ♦ – 10%; * – differences are statistically significant as compared with control, # – differences are statistically significant as compared with previous storage period; & – differences between infectious activity after storage at –20 and –80ºC are statistically significant ($p < 0.05$; $n = 5$).

At the same time, in preserving media with glycerol at –20ºC during one month of storage there was an infectious activity decrease in all samples, excluding those with concentration of 10% (Fig. 6). Whereas between 1 and 3 months of storage under these conditions there was a further decrease in virus infectious activity in the specimens with 2.5% glycerol. Between 3 and 6 months, a decrease in virus infectious activity occurred in the samples with 10% glycerol. Between 6 and 12 months, a significant decrease in infectious activity occurred in all media. The maximum virus safety at –20ºC for 12 months, 67% of initial activity, was ensured by medium with 5% glycerol. During storage for a month at –80ºC virus activity remained stable in media with glycerol in all concentrations under the test. Between 1 and 3 months under these conditions, a virus infectious activity decreased in medium with 10% glycerol, and between 3 and 6 months it remained stable. Between 6 and 12 months of storage, infectious activity decreased in all specimens. The maximum safety after 12 months, 77% of initial activity, was observed in the samples with 7.5% glycerol.

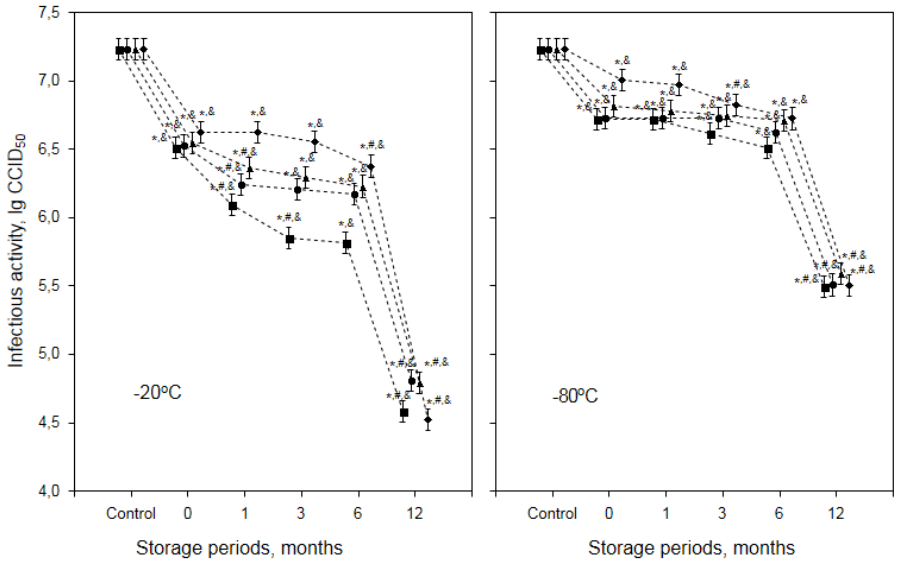

Fig 6: Dynamics of changes in infectious activity of L. Pasteur rabies virus strain during storage at temperatures of –20 and –80ºC in protective media with glycerol; ■ – 2.5%; ● – 5%; ▲ – 7.5%; ♦ – 10%; * – differences are statistically significant as compared with control, # – differences are statistically significant as compared with previous storage period; & – differences between infectious activity after storage at –20 and –80ºC are statistically significant (p < 0.05; n = 5).

Concerning the preserving media with DMSO, during virus storage at –20ºC, after one month, a virus activity decreased in the samples with 5 and 7.5% DMSO, after 3 months it decreased in all samples (Fig. 7). Between 3 and 6 months of storage, infectious activity did not change, and between 6 and 12 months, it decreased in all specimens. After 12 months of storage under these conditions, the maximum virus safety, 61% of the initial activity, was ensured by the medium with 5% DMSO. During storage at –80ºC similar to the abovementioned media at this temperature, the infectious activity of the virus remained stable in all samples for a month. Between 1 and 3 months, its decrease occurred in the medium with 10% DMSO. Between 3 and 6 months, activity remained stable in all media with DMSO, however, between 6 and 12 months, virus safety indices decreased in all the samples. After 12 months, the maximum virus safety, 79% of initial activity, was observed in specimens with 5% DMSO.

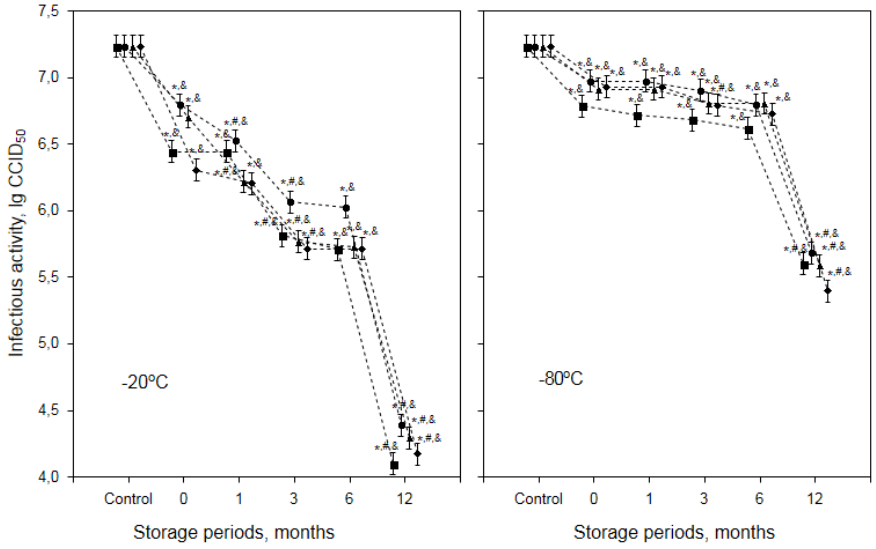

Fig 7: Dynamics of changes in infectious activity of L. Pasteur rabies virus strain during storage at temperatures of –20 and –80ºC in protective media with DMSO; ■ – 2.5%; ● – 5%; ▲ – 7.5%; ♦ – 10%; * – differences are statistically significant as compared with control, # – differences are statistically significant as compared with previous storage period; & – differences between infectious activity after storage at –20 and –80ºC are statistically significant (p < 0.05; n = 5).

And finally, in the media with gelatin at –20ºC, infectious activity of the virus remained stable for a month, and in the medium with 3% gelatin, it remained stable up to three months of storage, after which it decreased until the end of the observation period (Fig. 8). After 12 months, the virus safety at –20ºC was 58% of the initial virus activity in media with both studied gelatin concentrations. At the temperature of –80ºC the infectious activity in samples with gelatin did not change within 6 months. After 12 months of storage at this temperature in media with gelatin 79–80% of initial virus infectious activity remained.

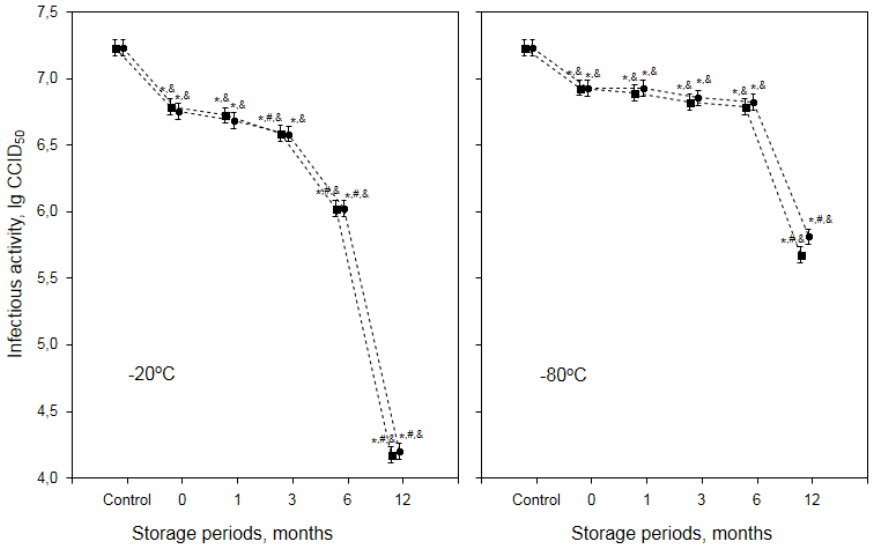

Fig 8: Dynamics of changes in infectious activity of L. Pasteur rabies virus strain during storage at temperatures of –20 and –80ºC in protective media with gelatin; ■ – 1%; ● – 3%; * – differences are statistically significant as compared with control, # – differences are statistically significant as compared with previous storage period; & – differences between infectious activity after storage at –20 and –80ºC are statistically significant ($p < 0.05$; $n = 5$).

3.4 Results Analysis

The obtained results indicate that despite the differences in the structure of viruses and cells, the rabies virus response to freezing conditions and temperature conditions of storage corresponds to the basic principles of generally accepted concepts of cryo-damage and cryoprotection of cells, in particular, two-factor and multifactor theories of cryoinjuries [[20], [21]]. According to these theories, the safety of biological objects in the process of low-temperature preservation largely depends on the speed and mode of cooling, the presence of cryoprotective substances in the preserving medium, and storage temperature. In the study, the virus samples were cooled at uncontrolled slow rates in freezers with temperatures of –20 and –80ºC. Considering the difference in temperature gradients, it can be argued that the cooling rates in the chamber with the temperature of –80ºC was higher. The values of the virus infectious activity after cooling to –80ºC were higher than after cooling to –20ºC. But there is no the reason to say that this cooling rates was more gently, since we determined the virus safety after cooling to –20 and –80 ºC a week after it was placed in cooling chambers. Therefore, for several days at –20ºC the virus was affected by the damaging physicochemical factors, which will be discussed below.

The temperature range from –10 to –80ºC refers to the crystallization zone of chilled and supercooled water [[22]]. Taking into account the composition of the used preserving media and the values of eutectic temperatures for the solutions of various electrolytes and cryoprotectants [[23]], it can be concluded that the virions during storage at –20ºC were constantly in the liquid phase in microchannels between ice crystals and were exposed to a complex of damaging factors: hyperconcentration of the salts and other components, changes in the pH of the medium, dehydration of macromolecules, disturbances in intermolecular interactions (“solution effects”) [[24], [25]]. In addition, under the low temperatures action, loosening and aggregation of proteins are possible [[23]], for rabies virus this process may involve RNA-dependent RNA polymerase. With an extension in storage periods at –20ºC the “time recrystallization” occurred. It comprises a further ice crystals growth with an increase in the salts concentration and other components and, consequently, an intensification of the “solution effects”. This led to the sharp decrease in the virus infectious activity between 6 and 12 months of storage. Higher virus preservation rates at –20ºC in media containing protective substances indicate that cryoprotectants, which have low eutectic temperatures and bind a part of water, reduced the eutectic point in stored samples compared to the preserving medium without cryoprotectants.

In the samples stored at –80ºC, the same physicochemical factors affected the virus. Increasingly, they caused damage virions of at the stages of cooling-heating [[10]]. Taking into account the fact that all the preserving media used in the study were multicomponent systems with structural heterogeneity, it is obviously, that at temperature of –80ºC the liquid microphases containing liquid solutions of salts and cryoprotectants remained in these media. These hyperconcentrated solutions damaged the virions during storage at –80ºC. By analyzing the curves of decrease in infectious activity after storage during the first six months and between 6 and 12 months, we assume that two processes took place. The first one is the direct death of virions during storage due to low temperature and the "solution effects". The second one is the accumulation of non-lethal and sublethal cryoinjuries in virions. These damages are implemented at the stage of heating with additional exposure of damaging physicochemical factors during thawing of the samples.

The obtained cryoprotective effect resulting from adding into the growth medium both penetrating cryoprotectants (DMSO, glycerol) and extracellular (sucrose, gelatin, sodium alginate) indicates that, upon freezing and low-temperature storage of viruses, the protective effect of cryoprotective substances is associated, on the one hand, with their hydration properties, on the other hand, with stabilization of the capsid and supercapsid of the virions.

IV. CONCLUSIONS

1. It was established that the infectious activity indices of rabies virus stored during the year at –80ºC were higher than at –20ºC, the differences were statistically significant. At temperature of –80ºC, it was possible to maintain up to 82% of the L. Pasteur strain activity, at the temperature of –20ºC we succeeded to maintain up to 71% of its activity.
2. It has been shown that during storage at –20 and –80ºC lethal damages of virions are caused by temperature and combination of physicochemical factors associated with the salts concentration and other components of the preserving medium.
3. The virus infectious activity of both strains stored at –80ºC was established to be higher than that at –20ºC. At –80ºC, we succeeded to maintain up to 88% of L. Pasteur strain activity and up to 90% of CVS strain activity.
4. During storage at –20 and –80ºC within a year (observation period), the highest safety parameters of L. Pasteur rabies virus strain were provided with preserving media based on growth medium (DMEM with 0.1% BSA) without additives and with sucrose, gelatin, glycerol and DMSO.
5. Since media with gelatin, glycerol and DMSO cannot be used due to regulatory requirements, the preserving media based on DMEM with 0.1% BSA, both without cryoprotectants and with the addition of 2.5-10% sucrose, could be recommended for use in manufacture.

ACKNOWLEDGMENTS

We would like to thank the management of the PJSC "Pharmstandard-Biolik" for providing the opportunity to accomplish this research. As well the authors are grateful to the team of the Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine for their methodical and practical assistance in performing of cryobiological experiments.

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Viktoriia Varianytsia. “Protective Media for Storage of L. Pasteur Rabies Virus Strain at Different Temperatures”. IOSR Journal of Pharmacy (IOSRPHR), vol. 9, no. 10, 2019, pp. 09-18.