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Cryopreservation of primary trypsinized fibroblast cells of chicken embryos using various cryoprotectants

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SUMMARY
Cryopreservation is the optimal way to store cells at ultra-low temperatures. Cryoprotectants are added to cell culture suspension to reduce cell death due to exposure to low temperatures. Cryoprotective media contain combinations of various cryoprotectants. Ethylene glycol, glycerin, dimethyl sulfoxide, sucrose, dextran, propylene glycol, albumin, polyvinylpyrrolidone and blood serum can be used as cryoprotectants. For cryopreservation it is necessary to select a cryoprotectant that ensures the highest survival of cells after storage and thawing. The paper presents the results of experiments on comparing the effectiveness of dimethyl sulfoxide, ethylene glycol and glycerin in cryopreservation of primary trypsinized chicken embryo fibroblasts. As a result of cell suspension equillibration (incubation at room temperature) with serum and the specified cryoprotectants at different concentrations, the suspension variants containing different cryoprotectant and serum ratios were selected for freezing. Previously, it was found that after 12 months of observation, when using dimethyl sulfoxide as a cryoprotectant, the largest number of surviving cells (46%) was observed in a suspension containing 20% fetal serum and 10% dimethyl sulfoxide. The amount of surviving cells if 10% fetal serum and 5% ethylene glycol were included in the cryoprotective mixture was slightly lower and amounted to 36% after 12 months of observation. Glycerin is shown to have weak protective properties as regards chicken embryo fibroblast cells. After 8 months of storage, the amount of surviving cells in a suspension containing 10% serum and 5% glycerin was 22%, no live cells were found in this mixture if stored longer. The proliferative properties of cells and their sensitivity to viruses remained within the 12 months of the experiment.

Keywords: cryopreservation, chicken embryo fibroblast cells, cryoprotectants

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Криоконсервирование первично трипсинизированных клеток фибробластов эмбрионов кур с использованием разных криопротекторов

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РЕЗЮМЕ

Криоконсервирование является оптимальным способом хранения клеток при сверхнизких температурах. Для уменьшения гибели клеток от воздействия низких температур к суспензии клеточной культуры добавляют криопротекторы. Сочетания различных криопротекторов образуют криозащитные среды. В качестве криопротекторов могут быть применены этиленгликоль, глицерин, диметилсульфоксид, сахароза, декстран, пропиленгликоль, альбумин, поливинилпирролидон и сыворотка крови. При проведении работ по криоконсервированию необходимо подобрать криопротектор, обеспечивающий наибольшую выживаемость клеток после хранения и оттаивания. В данной статье представлены результаты экспериментов по сравнению эффективности диметилсульфоксида, этиленгликоля и глицерина при криоконсервировании первично трипсинизированных клеток фибробластов эмбрионов кур. В результате эквилибрации клеточной суспензии (инкубирования при комнатной температуре) с сывороткой и указанными криопротекторами разных концентраций для замораживания были выбраны варианты суспензий, содержащие различные соотношения криопротекторов и сыворотки. Ранее было установлено, что по истечении 12 месяцев наблюдения при использовании в качестве криопротектора диметилсульфоксида наибольшее количество выживших клеток (46%) наблюдалось в суспензии, содержащей 20% фетальной сыворотки и 10% диметилсульфоксида. Количество выживших клеток при наличии в составе криозащитной смеси 10% фетальной сыворотки и 5% этиленгликоля было несколько ниже и составило 36% по истечении 12 месяцев наблюдения. Было показано, что глицерин обладает слабыми протективными свойствами по отношению к клеткам фибробластов эмбрионов кур. Спустя 8 месяцев хранения количество выживших клеток в суспензии, содержащей 10% сыворотки и 5% глицерина, составило 22%, при последующем хранении живых клеток в данной смеси не выявляли. Пролиферативные свойства клеток и чувствительность их к вирусам сохранялись на протяжении 12 месяцев эксперимента.

Ключевые слова: криоконсервирование, клетки фибробластов эмбрионов кур, криопротекторы

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INTRODUCTION

Cryopreservation is widely used for long-term storage of live cells, tissues, organelles, organs and whole organisms at ultra-low temperatures for a long period of time (from several months to several years). To be more specific, cryopreservation is the optimal way to store cell cultures of coelenterates [1] and other live organisms, mammal sperm and embryos, etc. A number of works have also been carried out to improve methods of freezing fish reproductive material [2, 3]. In recent years, cryopreservation methods of human cells and tissues were developed and updated. Thus, the manual cryopreservation method for erythrocytes was improved at the Federal State Budget Institution "Russian Research Institute of Hematology and Transfusiology of the Federal Medical and Biological Agency" (St. Petersburg) [4]. Japanese researchers developed a method for cryopreservation of human corneal endothelial cell culture [5].

Developments on cryopreservation of animal cell cultures and study of the biological properties of frozen cells are also important. Researchers of FGBI "ARRIAH" (Vladimir) developed methods for cryopreservation of continuous BHK-21 cell culture, primary trypsinized culture of chicken embryo fibroblast cells (CEF) containing Marek's disease virus, and primary culture of porcine bone marrow cells [6–8]. Specialists of the Federal State Budgetary Scientific Institution "Federal Center for Toxicological, Radiation, and Biological Safety" (Kazan) conducted studies of the biological properties of the transplanted bovine lung embryonic cell culture (LEK) subjected to long-term cryopreservation [9]. In addition, the results of the study on cryopreservation of broiler chicken embryo fibroblast cells were published [10].

An important aspect of cell culture cryopreservation is optimal selection of a cryoprotectant – a substance that protects cells from the freezing damage [6, 11]. There are two types of cryoprotectants: penetrating ones that easily penetrate into cells (dimethyl sulfoxide – DMSO, glycerin, ethylene glycol, propylene glycol), and non-penetrating ones that ensure external protective environment for cells (sucrose, dextran, albumin, polyvinylpyrrolidone) [11–14]. A cryoprotectant is often used in combination with stabilizers, commonly with blood sera [6–8, 13]. It is known about the use of a mixture of embryonic serum, DMSO and gold nanoparticles as a cryoprotective medium for human fibroblast cells [15]. Development of cryopreservation methods for different types of cell cultures suggested a high degree of cell survival (primary cells – about 58%, continuous cells – more than 90%) after freezing [2, 12]. There are a lot of combinations of preservation media. A mixture of cell suspension, serum and cryoprotectant is most commonly used in practice [6–8].

CEF cell culture is of great practical importance in virology and biological production, therefore, searching new, optimal combinations of preservation media during cell freezing remains relevant. Previously, some works on

selecting the optimal combination of DMSO and serum for cryopreservation of primary trypsinized CEF cells were carried out [16], however, no similar studies were conducted for other cryoprotectants.

The aim of this paper was to study the protective properties of various cryoprotectants during cryopreservation of primary trypsinized CEF cells and compare their effectiveness. At the same time, such indicators as cell survival after storage at ultra-low temperatures, proliferative properties and cell susceptibility to viruses after freezing were evaluated.

Based on the aim, the following objectives were set: selection of cryoprotectant-stabilizer combination that is not toxic to cells; study of cryoprotectant protective properties for freezing CEF cell suspension, as well as study of cell culture proliferative properties and sensitivity to viruses after freezing.

MATERIALS AND METHODS

Selection of the optimal combination of serum and cryoprotectants added to the cell suspension. CEF cell suspension for freezing was prepared according to previously developed methods [16, 17]. DMSO (Carl Roth, Germany), glycerin (JSC "Himreactiv", Russia) and ethylene glycol (JSC "Himreactiv", Russia) were used as cryoprotectants. Combinations of mixtures containing the cell suspension, the specified cryoprotectants and the fetal serum (BioClot, Brazil) used as a stabilizer were prepared. Table 1 shows variants of cryoprotective mixtures, including different combinations of cryoprotectants and fetal serum (as a percentage in the suspension volume).

Afterwards the cell mixture was equilibrated (incubated at a room temperature) with cryoprotectants and a serum, the suspensions prepared for cryopreservation were subsequently placed for storage.

Assessment of cell viability after thawing. This step was carried out according to the previously developed method [16].

Determination of cryopreserved CEF cell sensitivity to viruses. The cell cultures under study were infected with the reference strain of the avian leukosis virus ALV-J ADOL-HCI-P7 after 3rd passage (Avian Disease and Oncology Laboratory, USA). After the incubation period, the virus accumulation was determined by the enzyme immunoassay (ELISA) based on the concentration of virus-specific protein p27 using a commercial ProFLOK ALV Plus Ag kit (Zoetis, USA) according to the manufacturer's instructions [16].

Table 1
Samples of CEF cell suspension supplemented with cryoprotective agents

	10% serum				20% serum			
DMSO content	5%	10%	15%	20%	5%	10%	15%	20%
Glycerin content	5%	10%	15%	20%	5%	10%	15%	20%
Ethylene glycol content	5%	10%	15%	20%	5%	10%	15%	20%

RESULTS AND DISCUSSION

The work on comparing the effectiveness of various cryoprotectants and optimizing the conditions for CEF cell cryopreservation included the following steps: selection of optimal concentrations of cryoprotectants in a preservation mixture; study of viability and proliferative properties of cells after storage at minus 150 °C for 12 months.

To perform the first step, the equilibration of CEF cell suspensions containing the above specified combinations of cryoprotective agents was carried out (Table 1). The cell concentration in the initial cell suspension was $(1.10 \pm 0.01) \times 10^7$ cells/cm^3. The test results are shown in Table 2.

The test results showed that the highest cell survival (70–100%) was observed for the following variants of cryopreservation suspensions:

1) 10% serum and 5% DMSO (97% of surviving cells);
2) 10% serum and 10% DMSO (97% of surviving cells);
3) 10% serum and 15% DMSO (100% surviving cells);
4) 20% serum and 5% DMSO (97% of surviving cells);
5) 20% serum and 10% DMSO (100% surviving cells);
6) 20% serum and 15% DMSO (89% of surviving cells);
7) 10% serum and 5% glycerin (90% of surviving cells);
8) 10% serum and 5% ethylene glycol (74% of surviving cells).

Table 2
Cell concentration in suspensions for cryopreservation after equilibration, cells/cm^3 ($n = 3$)

10% serum				
Cryoprotectants	5%	10%	15%	20%
DMSO	$\mathbf{(1.07 \pm 0.06) \times 10^7}$	$\mathbf{(1.07 \pm 0.06) \times 10^7}$	$\mathbf{(1.10 \pm 0.00) \times 10^7}$	$(6.67 \pm 0.30) \times 10^6$
Glycerin	$\mathbf{(1.00 \pm 0.10) \times 10^7}$	$(7.20 \pm 0.26) \times 10^6$	$(6.23 \pm 0.32) \times 10^6$	$(3.26 \pm 0.38) \times 10^6$
Ethylene glycol	$\mathbf{(8.17 \pm 0.15) \times 10^6}$	$(7.60 \pm 0.30) \times 10^6$	$(7.60 \pm 0.30) \times 10^6$	$(6.70 \pm 0.17) \times 10^6$
20% serum				
	5%	10%	15%	20%
DMSO	$\mathbf{(1.07 \pm 0.06) \times 10^7}$	$\mathbf{(1.10 \pm 0.00) \times 10^7}$	$\mathbf{(0.98 \pm 0.10) \times 10^7}$	$(7.20 \pm 0.46) \times 10^6$
Glycerin	$(8.17 \pm 0.29) \times 10^6$	$(5.63 \pm 0.47) \times 10^6$	$(3.87 \pm 0.30) \times 10^6$	$(2.60 \pm 0.36) \times 10^6$
Ethylene glycol	$(6.90 \pm 0.00) \times 10^6$	$(6.80 \pm 0.17) \times 10^6$	$(6.03 \pm 0.15) \times 10^6$	$(6.60 \pm 0.26) \times 10^6$

The number of surviving cells was less than 70% in the other samples.

Most of the above samples (6 samples) included various concentrations of DMSO and fetal serum. After CEF cells were equilibrated with ethylene glycol and glycerin, one sample from each group was selected for further studies. The listed variants of the cell suspension were selected to study the CEF cell viability during storage at a temperature of minus 150 °C. Aliquots of cryopreservation mixtures containing cell suspensions, cryoprotectants and fetal serum in a volume of 4 cm^3 were placed in a foam thermal container and put for storage in a low-temperature freezer at minus 150 °C [16]. This method ensured cooling of the suspension at a rate of about 1 °C/min [12]. Thawing, cell count and seeding into culture flasks were carried out at intervals of one month. This step lasted 12 months. The test results of cell survival determination following cryopreservation at minus 150 °C are shown in Table 3.

The previously conducted studies showed that the highest survival rate of primary trypsinized CEF cells ranged from 53 to 71% [16]. After 12 months of storage, the maximum number of surviving cells (46%) was observed in case of a cell suspension containing serum in the amount of 20% and DMSO in the amount of 10% of the total volume. In a suspension containing serum and ethylene glycol at concentrations of 10% and 5%, respectively, the number of surviving cells decreased to 36%, however, this parameter was higher as compared to other samples containing various combinations of DMSO and serum. The number of live cells in the other suspensions containing DMSO and fetal serum decreased to 18–26% after 12 months of storage. The number of live cells in a suspension sample containing 10% serum and 5% glycerin decreased to 22% within 8 months. No live cells were detected in this sample after 9 months of storage. The smallest number of surviving cells was observed in the sample containing 10% serum and 15% DMSO. After one month of storage 24% of live cells were detected in that sample, the percentage of surviving cells decreased to 19% within the next 4 months of storage. No live cells were found in this mixture after 6 months of storage.

Subsequently, the proliferative properties of cryopreserved CEF cells were studied. After defrosting and counting, each sample cells were diluted with growth medium to a concentration of 600 thousand cells/cm^3 and seeded into culture flasks. The monolayer was daily observed under microscope, the duration of formation and the flask surface coating estimated in percentage were recorded [16].

Table 3
Survivability of CEF cells after storage at minus 150 °C using different cryprotectant samples ($n = 3$)

Storage period, months	10% serum and 5% DMSO	10% serum and 10% DMSO	10% serum and 15% DMSO	20% serum and 5% DMSO	20% serum and 10% DMSO	20% serum and 15% DMSO	10% serum and 5% glycerin	10% serum and 5% ethylene glycol
Before storage	10.0 ± 0.1	13.0 ± 0.1	9.0 ± 0.2	11.0 ± 0.3	8.0 ± 0.1	12.0 ± 0.3	8.0 ± 0.1	8.0 ± 0.1
1	3.3 ± 0.4 (33%)	4.9 ± 0.3 (37%)	**2.9 ± 0.2 (24%)**	4.2 ± 0.4 (38%)	**5.2 ± 0.3 (65%)**	5.7 ± 0.4 (47%)	**2.7 ± 0.2 (34%)**	**4.3 ± 0.2 (52%)**
2	2.9 ± 0.3 (29%)	4.3 ± 0.4 (33%)	2.9 ± 0.2 (24%)	4.0 ± 0.3 (36%)	4.9 ± 0.1 (61%)	4.9 ± 0.3 (41%)	2.5 ± 0.2 (31%)	3.5 ± 0.5 (43%)
3	2.9 ± 0.1 (29%)	4.0 ± 0.4 (31%)	2.1 ± 0.2 (23%)	4.0 ± 0.2 (36%)	4.6 ± 0.1 (57%)	4.8 ± 0.5 (40%)	2.5 ± 0.1 (31%)	3.3 ± 0.5 (41%)
4	2.8 ± 0.3 (28%)	3.0 ± 0.4 (23%)	2.1 ± 0.1 (23%)	4.0 ± 0.3 (36%)	4.3 ± 0.2 (54%)	4.2 ± 0.4 (35%)	2.4 ± 0.1 (30%)	3.3 ± 0.2 (41%)
5	2.6 ± 0.4 (26%)	3.0 ± 0.2 (23%)	**1.7 ± 0.3 (19%)**	4.0 ± 0.2 (36%)	4.3 ± 0.2 (54%)	4.1 ± 0.4 (34%)	2.1 ± 0.1 (26%)	3.2 ± 0.2 (40%)
6	2.5 ± 0.2 (25%)	3.0 ± 0.3 (23%)	0	4.0 ± 0.3 (36%)	4.2 ± 0.1 (52%)	4.0 ± 0.3 (33%)	2.1 ± 0.3 (26%)	3.1 ± 0.4 (39%)
7	2.3 ± 0.2 (23%)	3.0 ± 0.2 (23%)	0	4.0 ± 0.2 (36%)	4.0 ± 0.2 (50%)	4.0 ± 0.1 (33%)	2.0 ± 0.3 (25%)	3.0 ± 0.2 (37%)
8	2.2 ± 0.1 (22%)	3.0 ± 0.1 (23%)	0	3.8 ± 0.2 (34%)	4.0 ± 0.3 (50%)	3.9 ± 0.3 (32%)	**1.8 ± 0.3 (22%)**	3.0 ± 0.3 (37%)
9	2.0 ± 0.1 (20%)	3.0 ± 0.3 (23%)	0	3.5 ± 0.4 (32%)	3.9 ± 0.2 (49%)	3.9 ± 0.1 (32%)	0	3.0 ± 0.1 (37%)
10	1.8 ± 0.1 (18%)	3.0 ± 0.1 (23%)	0	3.5 ± 0.2 (32%)	3.9 ± 0.1 (49%)	3.7 ± 0.3 (31%)	0	3.0 ± 0.1 (37%)
11	1.8 ± 0.3 (18%)	2.9 ± 0.4 (22%)	0	3.2 ± 0.2 (29%)	3.8 ± 0.2 (47%)	3.6 ± 0.1 (30%)	0	2.9 ± 0.2 (36%)
12	1.8 ± 0.1 (18%)	2.3 ± 0.4 (18%)	0	2.9 ± 0.3 (26%)	**3.7 ± 0.1 (46%)**	3.2 ± 0.3 (26%)	0	**2.9 ± 0.1 (36%)**

Figure 1 demonstrates the data obtained at 1, 3, 6, 9, 12 months of storage. During testing of samples containing 10% serum and 15% DMSO, as well as 10% serum and 5% glycerin, the flask surface monolayer coating did not exceed 50%. The degree of flask surface coating with a CEF cell monolayer was 90–100% in other cases within 1–11 months of storage. At the end of 12 months of storage this value was 80%. The obtained values are optimal for the adequate cell culture monolayer.

The formation time of the monolayer in all CEF cell samples was 2–3 days, which is the optimal time for this process.

The final stage of this work was to study the susceptibility of CEF cells to viruses after exposure to low temperatures. CEF cells stored at minus 150 °C were thawed and seeded into culture flasks for monolayer formation. At the same time, a primary trypsinized CEF cell culture was used as control. The formed monolayer was infected with the reference strain of avian leukosis virus ALV-J ADOL-HCI-P7. Cell suspension samples containing 10% serum and 15% DMSO, as well as 10% serum and 5% glycerin, were not used at this stage of work due to weak proliferative properties. At the end of the incubation period, the concentration of virus-specific protein p27 was estimated using sandwich ELISA.

Figure 2 demonstrates the data obtained at 1, 3, 6, 9, 12 months of storage of cell samples as an example. The standard deviation ranged from 0.2 to 0.4. The virus accumulation was observed both in the primary trypsinized CEF cell culture and in the monolayer obtained from suspension of cryopreserved CEF cells. The concentration of the virus protein was 5–8 μg/cm^3. Throughout the study, a slight decrease in the concentration of virus protein was observed in a number of samples compared to the primary trypsinized CEF cell culture. The minimal discrepancy between this parameter and the value obtained for the primary trypsinized CEF cell culture was observed in a sample containing 10% serum and 5% ethylene glycol.

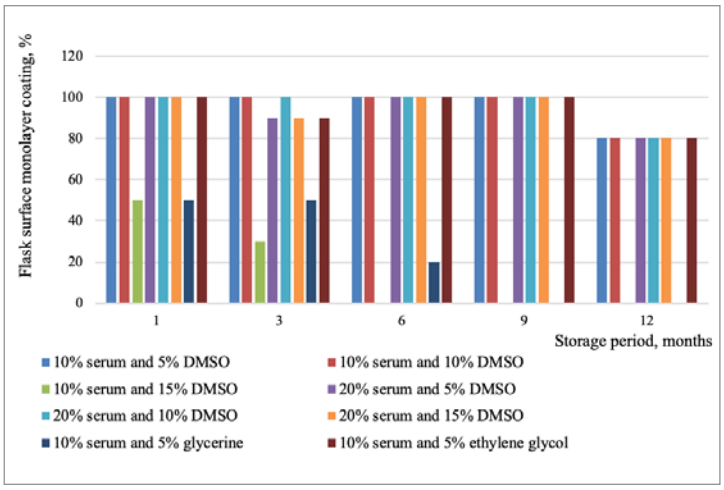

Fig. 1. Development (formation) of cell monolayer upon thawing

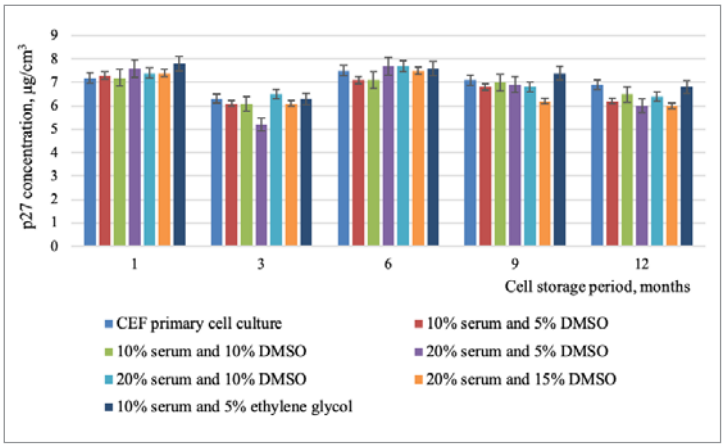

Fig. 2. Concentration of p27 viral protein after infection of CEF cell samples with ALV-J ADOL-HCI-P7 strain, μg/cm^3 (n = 3)

CONCLUSION

The study results revealed that the most optimal serum-cryoprotectant ratio for CEF cell suspension in deep freezing conditions is a combination of 20% fetal serum and 10% DMSO. The number of surviving cells in the cell suspension sample containing 10% fetal serum and 5% ethylene glycol was slightly lower, but it exceeded the same indicator in other samples. Glycerin added to the cell suspension did not provide sufficient cell protection from destructive effects of ultra-low temperatures. Proliferative cell properties and sensitivity to avian leukosis virus were preserved in the following cryoprotectant combinations: containing 10% bovine embryonic serum and 5, 10% DMSO, 20% bovine embryonic serum and 5, 10, 15% DMSO, as well as 10% bovine embryonic serum and 5% ethylene glycol. Cryoprotectant samples containing 10% serum and 15% DMSO, as well as 10% serum and 5% glycerin, showed the lowest protective property.

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