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ACTION OF PHYSICAL AGENTS ON BIOLOGICAL OBJECTS

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EFFECTS OF CRYOPROTECTIVE AGENTS ON OSMOTIC TOLERANCE LIMIT OF TESTICULAR INTERSTITIAL CELLS

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Background: Cryopreservation is a multistep process, which includes stages affecting biological material mechanically, osmotically and toxically. The use of cryopreservation of biological materials is cost-effective and affording long-term storage at cryogenic temperatures. It also guarantees the stability of the genetic component of cells and reduced contamination of the biological material.
Objectives: The objective of the research is to evaluate the effects of cryoprotective agents (CPAs) (dimethyl sulfoxide (DMSO), dextran (D40), hydroxyethyl starch, polyethylene glycols (PEG1500 and PEG400), and fetal bovine serum) and their combinations on the interconnection between the osmotic tolerance of testicular interstitial cells (ICs) and cryoprotection.
Materials and Methods: The osmotic tolerance limit (OTL) of ICs and the toxic effect of the CPA were investigated in the phosphate buffer saline based media of different osmolarities: isosmotic (300 mOsm), hypo-osmotic (225 mOsm), hyperosmotic (600 mOsm). Similar osmotic conditions can develop during cryopreservation of cells in the temperature interval from +4 to -30 °C.
Results: The indicators of cell survival after incubation in the media differed depending on osmolarities of incubation media. They were compared with the indicators obtained after cooling ICs to -30 °C followed by warming and CPA removal. We have shown that the non-toxic additive D40 increased the OTL of ICs in hypo-osmotic medium and decreased negative effects of DMSO on the cells. These effects were accompanied by high indicators of ICs survival obtained after cooling ICs to -30 °C with 100 mg/ml D40 and 0.7 M DMSO.
Conclusions: These results unveil the mechanisms of cryoprotection of 0.7DMSO+D40 and partially explain the superiority of 0.7DMSO+D40 media shown in our previous works compared with other investigated media. Understanding the mechanisms of cryodamage and cryoprotection of 0.7DMSO+D40 paves a way toward the development of new serum-/xeno-free cryoprotective compositions and improvement of cryopreservation protocols for cell suspensions that include many types of cells. Further studies are required to reveal the effects of DMSO on membranes and intracellular metabolic processes.
KEY WORDS: osmotic tolerance; toxicity; dimethyl sulfoxide; dextran; hydroxyethyl starch; polyethylene glycol; mechanisms of cryoprotection.

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Preservation of living cells is a central technology that brings cell-based products to market on-demand. The use of cryopreservation of biological materials is cost-effective and affording long-term storage at cryogenic temperatures. It also guarantees the stability of genetic content of cells and reduced contamination of the biological material [1]. Our previous works were devoted to the development of serum-/xeno-free cryoprotective compositions for testicular interstitial cells (ICs) and to investigation of the mechanisms of their cryoprotective effects [2]. The biological material of ICs is an example of a cellular suspension, which includes different types of cells: Leydig cells and cells of the immune system. Such cellular suspensions can be used for the creation of germplasm banks, for preservation of endangered species, breeds and lines, as well as in reproductive technologies [3–7]. The use of cryoprotective media that include combinations of cryoprotective agents (CPAs) was shown to be relevant for cellular suspensions consisting of different types of cells [8]. Moreover, the development of serum-/xeno-free cryoprotective compositions may help to avoid problems connected with the use of serum such as transmission of infections to the biological material and instability of composition of cryoprotective media from batch-to-batch [9–11]. We have shown that a serum-free medium that included 0.7 M DMSO and 100 mg/ml dextran (M.m. 40 kDa) (0.7DMSO+D40) had an exceptional property for preservation of ICs. Some indicators of ICs survival were even higher than in the best serum-containing media that was supplemented with 1.4 M DMSO and 10% fetal bovine serum (FBS). If D40 was replaced by other polymeric compounds, such as hydroxyethyl starch (HES), polyethylene glycol (PEG), polyvinylpyrrolidone, the cryopreservation of IC was less effective (with HES) or non-effective (with PEG). 0.7DMSO+D40 was able to suppress intracellular crystallization, salt eutectic crystallization/melting and recrystallization [12]. The medium promoted amorphous phase formation and changed the shape and size of water crystals in a way that increased IC survival [2, 13]. However, the osmotic effects of the cryoprotective additives and their cytotoxicity were less explored.

Cryopreservation is a multistage process, which includes isolation of cells, addition of cryoprotective media, cooling, storage, warming, removal of cryoprotective media, and recovery of biological material. Any of the mentioned stages can contribute to a loss of cells. Addition of CPAs before cooling and their removal after warming can cause damaging cell volume excursions [14]. CPA addition as well as extracellular crystallization result in the formation of a hyperosmotic environment that causes cell shrinkage. The shrinkage itself can be damaging as it leads to membrane deformations, stresses, loss of integrity, increased permeability to extracellular ions, distorted interaction of the membrane and cytoskeleton [15, 16], and cell surface loss [14, 17–19]. On warming and CPA removal cells can swell. The swelling can cause membrane rupture, especially, if cooling was accompanied by the influx of normally extracellular ions into the cells and/or the membrane surface reduction [18, 20, 21]. Of note, the processes generally occur in the temperature interval from positive temperature to about -30 °C and this is the temperature interval where main bulk water crystallization/melting takes place [13]. The safety range of cell volume excursions is known as the OTL [14]. The plasma membrane damage is a stochastic phenomenon affecting a variable number of cells but allowing others to survive the expansion [22]. The osmotic injury is difficult to separate from the cytotoxic influence of CPA, which includes physical and chemical interaction of CPA with membranes, cytoskeleton and other cellular components. DMSO, for example, can interact with the membrane phospholipids and reduce membrane thickness [23], or promote the formation of pores [24]. Obviously, such DMSO effects will affect the OTL of the cells.

We supposed that, if the above-mentioned cryoprotective compositions differed by the type of additives, which resulted in different cryopreservation outcomes, they would also influence the osmotic tolerance of cells in hypo- and hyperosmotic conditions and their ability to

withstand different concentration of DMSO. If so, the cells' ability to survive during cryopreservation with the cryoprotective media could be partially explained by the stabilizing effect of the additives on the membrane integrity and their effect on DMSO cytotoxicity. Thus, we have investigated indicators of cell survival in hypo- and hyperosmotic conditions, showed the effect of additive on the indicators, and matched the data with the survival of cells after cooling to -30 °C. The chosen indicators of cells survival are the general cell survival, which reflected the number of cells whose membranes did not rapture, and the preservation of metabolic activity that, in addition, helped indicate the integrity of internal cellular processes and cellular structures after interaction with components (DMSO and polymers) of investigated solution. The objective of the research is to evaluate the effects of DMSO, polymers and serum on the interconnection between the OTL of ICs and cryoprotection.

MATERIALS AND METHODS

Testicular interstitial cell isolation

Interstitial cells were isolated from male Wistar rats. These animals were kept in the animal house of the Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine (Kharkiv). The experimental protocols followed the Guide for Care and Use of Laboratory Animals. They were approved by the Committee for Ethics in Animal Experimentation of the Institute for Problems of Cryobiology and Cryomedicine. The isolation was generally done as described [25]. Briefly, mature male rats were killed by cervical dislocation. Then, their bodies were sterilized by immersion in 75% ethanol for 5 min. Their testes were isolated and decapsulated. Blood vessels were trimmed. The content of the testes was placed in 15-mL centrifuge tubes with 4 ml of 0.2 mg/mL collagenase (type I) (Sigma-Aldrich, USA) and 0.1 mg/mL DNase I (Sigma-Aldrich, USA) in DMEM F12 (Biowest, France) for 15 min. The tubes were transferred to a thermostatic shaking water bath for 10 min (90 cycles/min, 34 °C). After that, 10 ml of pure precooled (+4 °C) PBS (300 mOsm) were added to each tube. Thus, the seminiferous tubule mass was obtained. It was filtered through a doubled 100-μm nylon mesh. The seminiferous tubules were discarded whereas the filtrates were centrifuged at 325 g for 3 min. The supernatants were discarded when the pellets were collected and resuspended in 10 mL PBS. The sedimentation operation was repeated. The concentration of ICs was adjusted to 4×10^7 cells/mL by PBS. If ICs were supposed to be cooled, DMEM F12 was added to the pellet instead of PBS after the last centrifugation and cell concentration adjustment was done with DMEM F12.

Osmotic tolerance limit measurements

The effects of DMSO, D40, HES, PEG1500, PEG400, FBS on the OTL of ICs were measured in the phosphate buffer saline (PBS) of different osmolarities: 225 (hypo-osmotic PBS), 300 (isosmotic PBS), 600 mOsm (hyperosmotic PBS). The PBSs were additionally supplemented with 0.7 M DMSO and/or one of the above-mentioned polymers (D40, HES, PEG1500, PEG400). Alternatively, the PBSs were added with 1.4 M DMSO and/or 10% (v/v) FBS. The stock solutions of DMSO, polymers and FBS in PBS were prepared in a way that, on mixing with IC suspension (1:1), gave their mentioned final concentrations and osmolarities. Therefore, the final concentration of cells supposed to be incubated also dropped to 2×10^7 cells/mL. The cells were then incubated for 40 min at 4 °C. The incubation media were replaced with isosmotic PBS by gradual dilution: five aliquots of PBS (200 μL) were added every 30 seconds to 1 ml of ICs, followed by addition of two 500 μL- and three 1 mL-aliquots. The cells were centrifuged at 325 g for 3 min and the supernatant was discarded. 5 mL of PBS was added and the procedure of sedimentation and supernatant removal was repeated again. After the last

centrifugation, the pellet was diluted with DMEM F12 to 1 ml. Finally, the indicators of cell survival: general cell survival and preservation of metabolic activity of cells were assessed.

Cooling/warming

The cryopreservation media were prepared similarly to the incubation media for the OTL measurements, however, DMEM F12 medium was used instead of PBSs as a basic solution. ICs were added to the cryopreservation media (1:1) in 1.8-mL cryocontainers (Nunk, Denmark) to reach the final concentration just before cooling. The final volume of cell suspension, thus, came to 1 mL and the concentration of ICs was 2×10^7 cells/mL. The cryocontainers were placed into the programmable freezer and cooled to -30 °C at a rate of 1 °C/min. A copper-constantan thermocouple was used to detect the temperature in the control sample. Once the final temperature was reached, the samples were kept at the set temperature of -30 °C in the freezer chamber for 7 minutes to reach the required final temperature. When the final temperature was reached, the cryocontainers were transferred to a water bath at 37 °C and warmed. The samples were kept in a water bath until the crystalline phase disappeared. The cryopreservation media were replaced with isosmotic PBS by gradual dilution as described above for the OTL measurements. The indicators of cell survival were measured.

General cell survival and preservation of metabolic activity measurements

A hemocytometer chamber was used for counting of ICs. The general cell survival was determined as a ratio of the number of all cells in a sample after incubation (or cryopreservation) to the number of cells before incubation (or cryopreservation) multiplied by 100%.

IC metabolic activity was determined after incubation or cryopreservation as described [2]. Briefly, 50 μL of 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich, USA) solution in PBS (5 mg/mL) was added to 500 μL of the IC suspension after incubation or cryopreservation. After that, the cells were incubated for 2 h at 35 °C. Then, ICs were sedimented at 325 g for 3 min. The supernatant was discarded. 450 μL of DMSO was added to the stained pellet and the samples were stirred. The samples were centrifuged after 5 minutes. The optical density of the supernatants was measured at 530 nm. The samples containing no cells were used as a reference. Metabolic activity preservation was expressed as the ratio of the optical density of a sample after incubation or cryopreservation to the optical density of a sample before incubation or cryopreservation multiplied by 100%.

Fluorescence measurements

For the spectrofluorometric analysis, ICs after incubation or cryopreservation with DMSO, followed by its removal, were placed in DMEM F12. The number of cells was adjusted to 2×10^6 cell/ml by diluting the cells with DMEM F12. A 50 μL-aliquot of probe O1O (synthesized in the Institute for Single Crystals, National Academy of Science, Kharkiv, Ukraine – [26]) stock solution in acetonitrile was added to 1 mL of ICs. The final probe concentration was 5×10^{-6} M. Then, the samples were incubated with the probe at 20 °C (room temperature) for 40 minutes before fluorescence measurements with spectrometer "PerkinElmer FL8500". Probe O1O emission was measured in the range of 340-550 nm, with an increment of 0.1 nm, the emission scan speed of 240 nm/min, the excitation wavelength of 330 nm, the excitation and emission slits of 5 nm.

The membrane location of fluorescent probe O1O: the area of glycerol backbones of phospholipids closer to the center of the lipid bilayer, the area of carbonyl groups of phospholipids and the area of hydrocarbon chains of phospholipids near the area of the carbonyl groups of phospholipids of the lipid membrane [27]. In the excited electronic state, the initial form of the probe (or so-called "normal" form: N^*-form) converts into photoproduct - tautomer

form (T^*-form) [28, 29]. The phototautomer has a long-wavelength fluorescence band, while the initial form of the probe emits in significantly shorter wavelengths [30]. The amount of the formed phototautomeric form depends on the physico-chemical properties of the probe microenvironment [28, 29]. Probe O1O has two-band fluorescence, hence, the ratiometric measurements are possible: T^*-form fluorescence intensity-to-N^*-form fluorescence intensity ratio can be used as a parameter. For instance, the I_{T*}/I_{N*} ratio increases when the polarity and/or hydrogen-bonding ability of the probe microenvironment decreases.

The fluorescence intensity in arbitrary units versus emission wavelength, were processed with Origin 2018 (OriginLab Corporation, Northampton, MA 01060, USA) and normalized with respect to native cells in pure DMEM F12. The fluorescence intensities of T^*- and N^*-form were detected at 480 and 390 nm, respectively, and used for the calculation of the actual values of the I_{T*}/I_{N*} ratio. The changes in the actual values of I_{T*}/I_{N*} were normalized to the samples without DMSO containing native cells.

Statistical analysis

Data were represented as a median, 25th percentile, 75th percentile, maximum and minimum. The minimal number of repetitions for each measurement was six. The data were ranked before the Newman–Keuls was used to reveal the differences between several groups and $p \leq 0.05$ was considered significant. The Dunnett's test was used to compare the samples having additives with samples incubated in pure PBS.

RESULTS AND DISCUSSIAN

Incubation in isotonic conditions

Incubation of ICs with D40, HES or FBS did not result in the significant cell loss (Fig. 1A) comparatively to PBS samples. Incubation with D40 or FBS also did not change the percentage of metabolically active cells in the samples (Fig. 1B), while in samples incubated with HES we observed significantly lower metabolic activity contrary to PBS variant. However, 0.7 and 1.4 M DMSO as well as their combinations with other additives lowered the indicators significantly. Interestingly, the presence of PEG400 in the media containing 0.7 M DMSO further decreased indicators of cell survival. Apart from PEG400, PEG1500 was less damaging to IC but its use with DMSO in 0.7DMSO+PEG1500 essentially decreased the metabolic activity of the samples (Fig. 1B) when the general cell survival was elevated compared with the sample containing DMSO only (Fig. 1A). 0.7DMSO+D40 and 0.7DMSO+HES had the same indicators of metabolic activity as the sample with 0.7 M DMSO but their indicators of general cell survival tended to be even higher (Fig. 1A and B).

Incubation in hypotonic conditions

Incubation in hypotonic PBS (225 mOsm) negatively affected both indicators of cell survival compared with correspondent solutions based on 300 mOsm PBS (Fig. 2A, Fig. 2B). The indicators of cell survival did not decrease in pure 300 mOsm PBS but 225 mOsm PBS lowered general cell survival and metabolic activity by about 65 and 50 % respectively. One exception is the PBS solution supplemented with PEG1500 where a slight increase was observed. However, D40 as well as PEG1500 significantly increased the metabolic activity in the samples compared with pure PBS (225 mOsm) (Fig. 2B). Typically, DMSO lowered the general cell survival (Fig. 2A) but addition of any polymer or FBS positively changed the indicators (Fig. 2A, Fig. 2B). Interestingly, the general cell survival in 0.7DMSO+D40 was even higher than in pure PBS (225 mOsm) (Fig. 2A). The metabolic activity of all 0.7 M DMSO containing media with/without polymers was comparable to pure PBS (except the significant

reduction in 0.7DMSO+PEG400), while the use of 1.4 M DMSO led to considerable inhibition of metabolic activity.

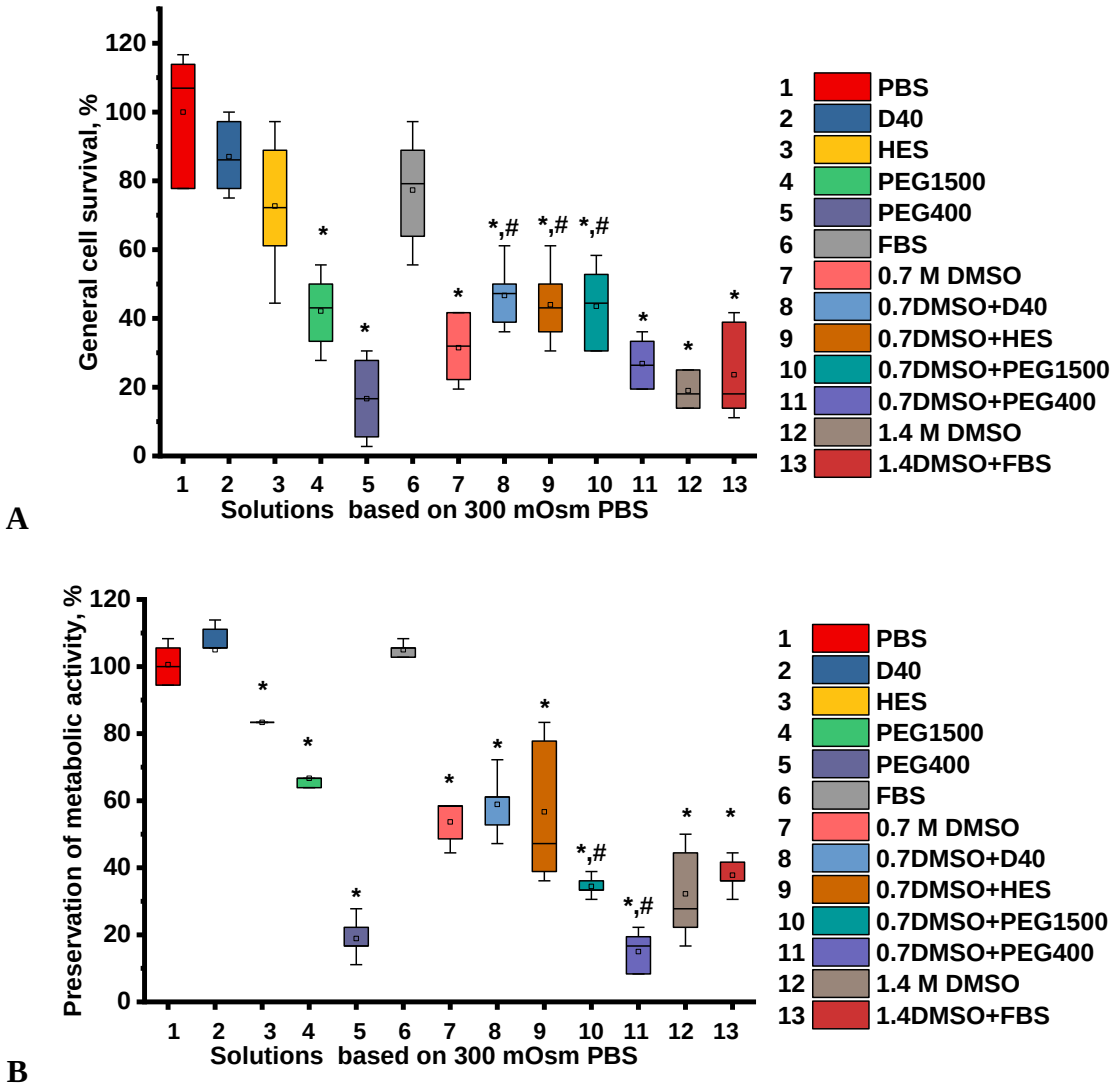


Figure 1. Indicators of IC survival after incubation in 300 mOsm PBS supplemented with DMSO and/or polymers or FBS: (A) general cell survival; (B) preservation of metabolic activity.
* – the indicators of cell survival were statistically different from pure PBS (300 mOsm) ($p \leq 0.05$);
# – the indicators of cell survival were statistically different from polymer- and serum-free PBS having the same DMSO concentration ($p \leq 0.05$).

Hypertonic conditions

The media based on 600 mOsm PBS also lowered indicators of general cell survival and their metabolic activity in most samples compared to the media based on isosmotic PBS (300 mOsm) by 40 and 20 % respectively (Fig. 3A, Fig. 3B). Polymers in the medium lowered either one or another indicator of cell survival (or both), except D40. 0.7DMSO alone had no effect neither on cell survival nor on metabolic activity (Fig.3A, Fig.3B) as well as its combination with D40, but 1.4 DMSO reduced both indicators significantly (Fig.3A, Fig.3B) compared to pure PBS (600 mOsm). PEG400 and FBS did not improve the outcome of incubation with DMSO, although FBS alone did not decrease the indicators of IC survival. It should be mentioned that PEG400 had the most prominent negative effect if both indexes are analyzed in hypertonic conditions.

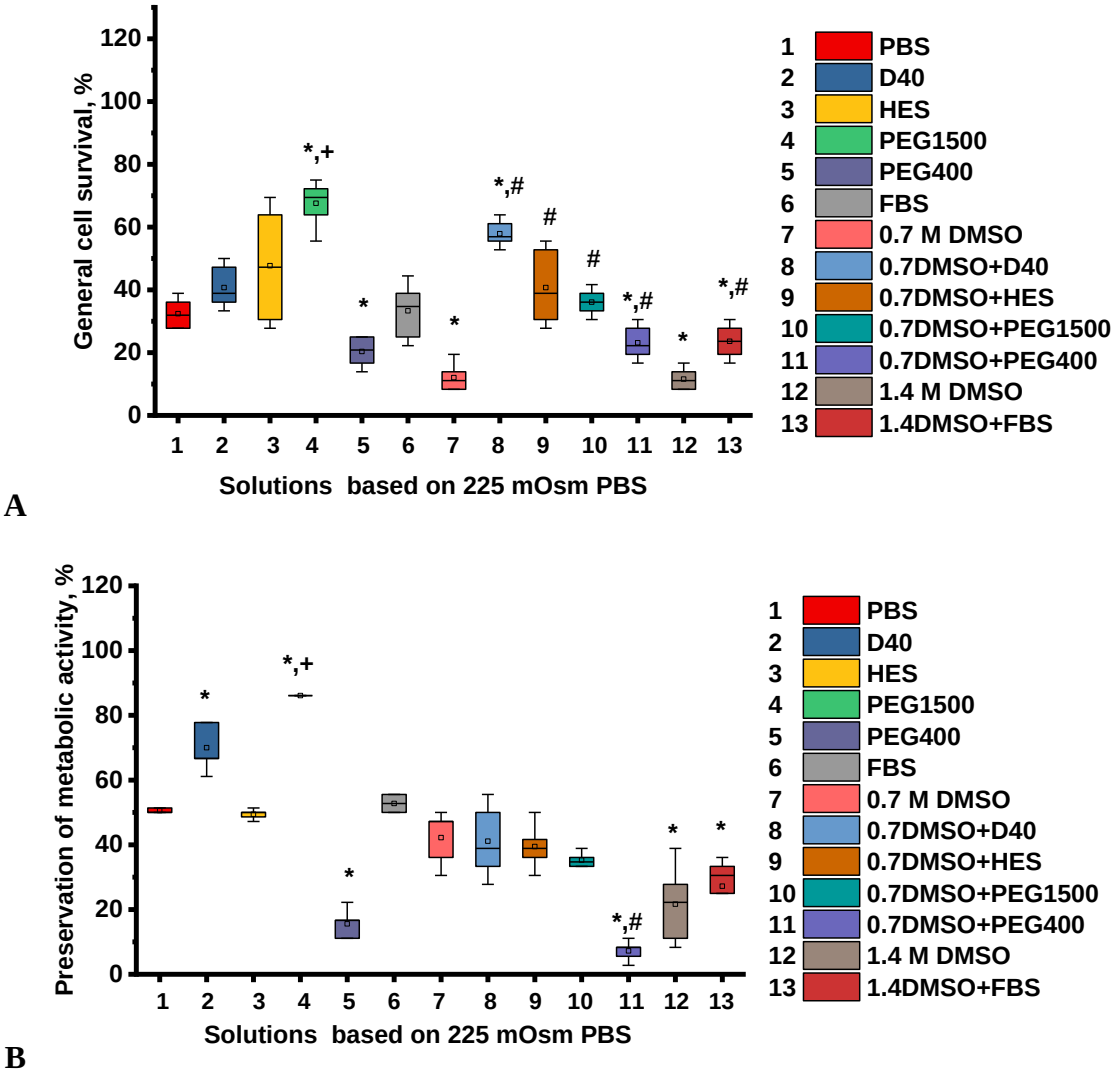


Figure 2. Indicators of IC survival after incubation in 225 mOsm PBS supplemented with DMSO and/or polymers or FBS: (A) general cell survival; (B) preservation of metabolic activity.
* – the indicators of cell survival were statistically different from pure PBS (225 mOsm) ($p \leq 0.05$);
# – the indicators of cell survival were statistically different from polymer- and serum-free PBS having the same DMSO concentration ($p \leq 0.05$);
+ – the indicators of cell survival were statistically higher than the same indicators of 300 mOsm PBS having identical concentrations and compositions of additives ($p \leq 0.05$).

Cooling/warming

The combinations of D40 or HES with 0.7 M DMSO significantly increased the general survival of ICs after cooling/warming (Fig. 4A). The increase also correlated with high metabolic activity in the samples (Fig. 4B). 0.7DMSO+D40 had best cryoprotective properties towards ICs. The use of FBS had a moderate impact on the survival of ICs: it did not increase the number of the surviving cell when combined with 1.4 M DMSO but significantly elevated the metabolic activity of the samples compared with the sample having 1.4 M DMSO alone (Fig. 4B). The use of 0.7DMSO+PEG1500 and 0.7DMSO+PEG1500 did not bring any advantage compared to the media supplemented by 0.7 M DMSO only.

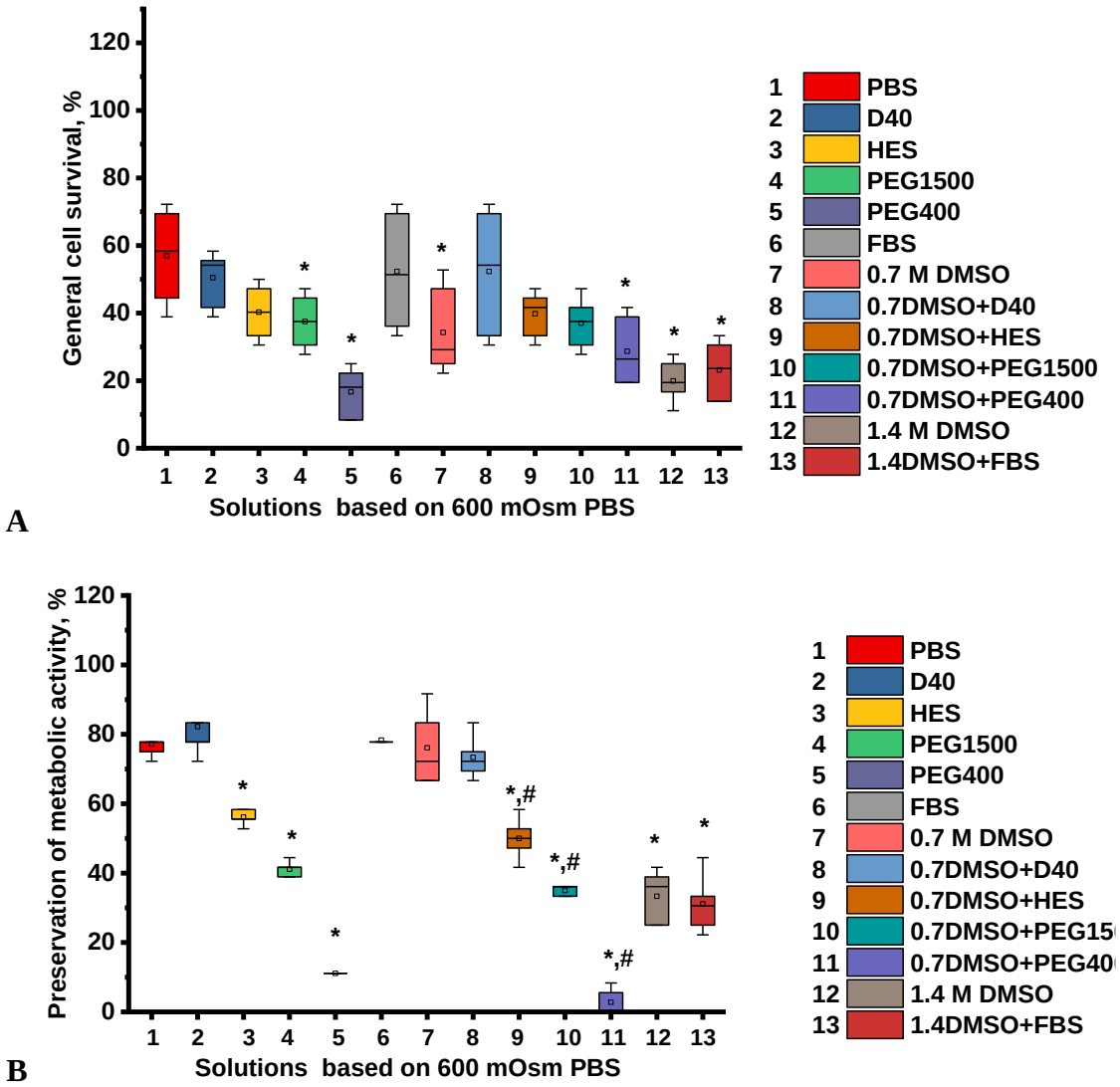


Figure 3. Indicators of IC survival after incubation in 600 mOsm PBS supplemented with DMSO and/or polymers or FBS: (A) general cell survival; (B) preservation of metabolic activity.
* – the indicators of cell survival were statistically different from pure PBS (600 mOsm) ($p \leq 0.05$);
# – the indicators of cell survival were statistically different from polymer- and serum-free PBS having the same DMSO concentration ($p \leq 0.05$).

Taking into account that DMSO is membrane permeable and able to change membrane physical-chemical properties [31], on the next stage of the study we estimated the influence of 0.7 M and 1.4 M DMSO on the state of phospholipid bilayer of ICs, using a fluorescent probe O1O (Fig. 5. A and B). Low-intensive short-wavelength band belongs to the N^*-form of the probe, while high-intensive long-wavelength band is attributed to emission of its T^*-form. It was found that, on comparison to the sample without additives, no considerable changes in the T^*-form fluorescence intensity-to-N^*-form fluorescence intensity ratio (i.e., I_{T*}/I_{N*} ratio) were observed at lower concentration of DMSO in both cases - after incubation and after cryopreservation: the I_{T*}/I_{N*} ratio was 0.98 (0.81; 0.99) and 1.01 (0.95; 1.07), respectively. On the contrary, at higher concentration of DMSO (1.4 M) a noticeable increase in I_{T*}/I_{N*} ratio ($p < 0.05$) was observed: 1.12 (1.12; 1.12) after incubation and 1.08 (1.06; 1.10) after cryopreservation.

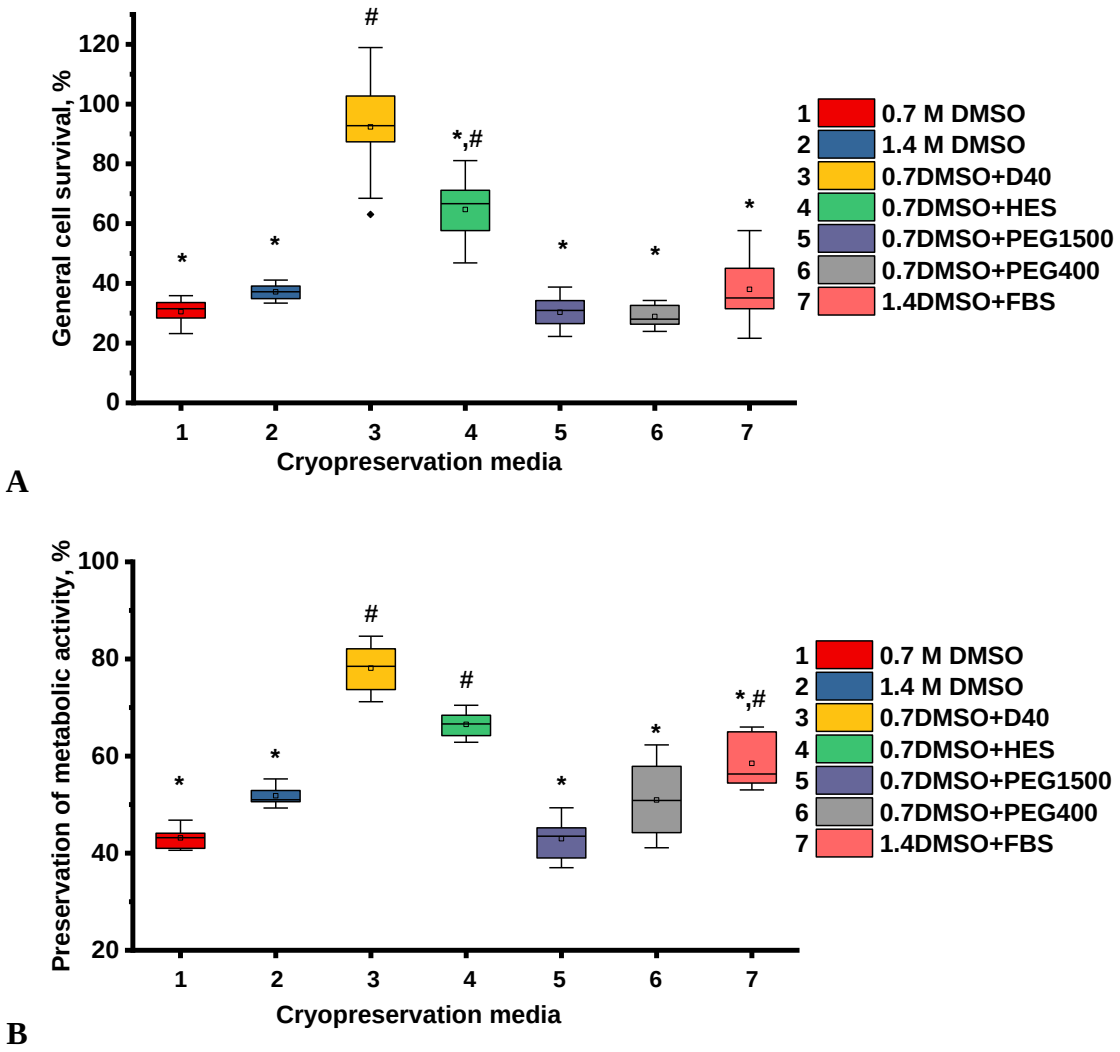
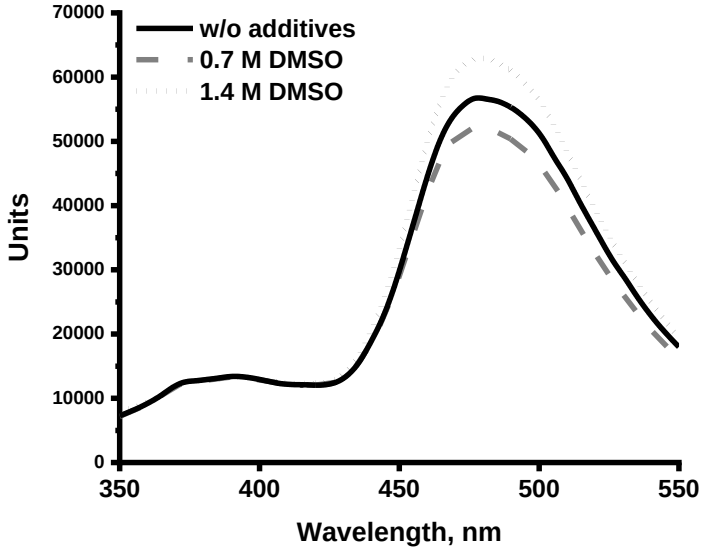


Figure 4. Indicators of IC survival after cooling/warming cycle to -30 °C with DMSO and/or polymers, or FBS: (A) general cell survival; (B) preservation of metabolic activity.
* – the indicators of cell survival were statistically different from 0.7DMSO+D40 ($p \leq 0.05$);
# – the indicators of cell survival were statistically different from the polymer- or serum-free media having the same concentration of DMSO.

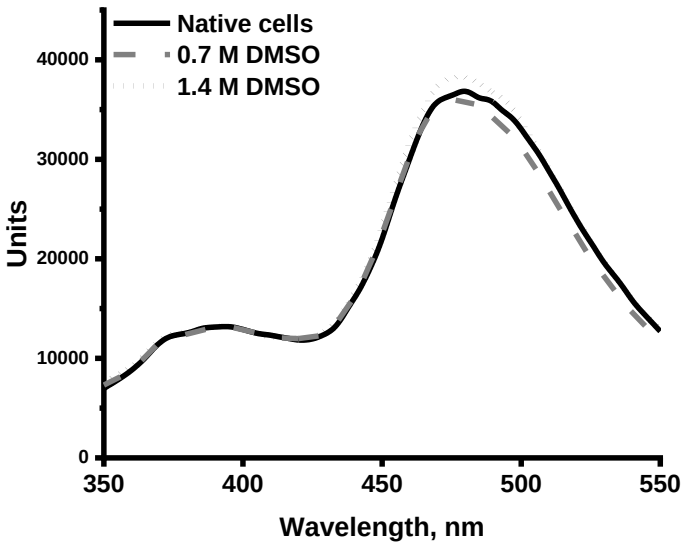
Cryopreservation of cell suspensions involves multiple steps, including cell isolation, the addition of cryopreservation media, equilibration with the media, cooling, storage, warming, removal of CPAs, and transferring the cells into new media for subsequent use. Certain stages expose cells to osmotic stress. For instance, during cooling, water crystallization creates hyperosmotic conditions around the cells, leading to water loss and cell shrinkage, which reduces cell volume. This shrinkage, combined with the toxic effects of highly concentrated solutes, can significantly lower the survival rate of the cells. Conversely, during warming, cells may experience the opposite effect, with changes in volume in the other direction.

The components of cryopreservation media can be conditionally subdivided into membrane permeable and impermeable. Permeable solutes such as DMSO when added can cause temporal cell shrinkage followed by restoration of cell volume to the initial one and, in some cases, by swelling [20, 21]. CPA removal can cause opposite changes: cell swelling followed by volume restoration. Elevated concentrations of conditionally impermeable solutes,

for example, Na^+, K^+, Cl^-, may lead to a persistent cell volume loss provided the membrane remains impermeable in such hypertonic media or the hypertonicity does not result in opening ion channels which can lead to the equilibration of ion concentration across the membrane. However, if the latter happens, the excessive concentration of the solutes in the cells may cause cell swelling and/or membrane damage when the cells are transferred back into isosmotic conditions, for example, during cryopreservation on warming and/or CPA removal. This effect is known as the post-hypertonic lysis and may contribute to cell loss.



A



B

Figure 5. Representative fluorescence spectra of probe O1O in cell-containing DMEM F12: (A) after incubation, followed by DMSO removal; (B) after cryopreservation, followed by DMSO removal. For better comparison, the spectra of the probe in the cells in the additive-free medium are presented on the graphs. To show the differences in the fluorescence intensities of the phototautomer forms, the spectra are normalized to the fluorescence intensity of the normal form of the sample with no additives.

Excessive shrinkage or swelling can cause damage to the cellular membrane and cell loss. Under the same deviation of osmolarity from the isosmolar condition, cell membranes are more sensitive to swelling which typically occurs in hypotonic media [32, 33]. This finding can be explained based on the theory of elasticity: stretching an oblong extended object (in our case the membrane in a hypotonic solution) is more difficult than bending it (or deforming the membrane on cell squeezing in a hypertonic solution) [17]. The stress provoked by hypotonicity can be accommodated by membrane rupture and cell death. In our experimentations, lowering osmolarity by 25 % (from 300 to 225 mOsm) resulted in a more dramatic decrease in the indicators of cell survival than increasing osmolarity by 100 % (from 300 to 600 mOsm). Similar observations were obtained [34]. Membrane stretching and bending may cause lateral separation of membrane lipids, which is carefully reviewed [17, 35, 36]. This effect is responsible for the increased permeability of the membrane and sensitivity to cooling/warming.

DMSO is common penetrating CPA able to form multiple interaction with water molecules, thus decreasing the amount of ice that can be formed on cooling at any given temperature, mitigating "solution effect", preventing excessive cell dehydration during formation of extracellular ice, increasing the amount of amorphous phase and making lipid phase transitions in cell membranes less cooperative [2, 12, 13, 31, 37]. These effects of DMSO promote the survival of many types of cells. However, it is known to be toxic for cells. Taking into account that polarity of DMSO is lower than that of water (dielectric permittivity of DMSO is 46.68 and DP of water is 81) and hydrogen-bonding ability of DMSO molecule is lower than that of water molecule (DMSO molecule has no proton-donating ability), one can suggest that the observed changes in the microenvironment of probe O1O at higher concentration of DMSO (1.4 M) may be caused by the solvation of the membrane with DMSO in the regions, where the probe locate: in the area of glycerol backbones of phospholipids closer to the center of the lipid bilayer, the area of carbonyl groups of phospholipids and the area of hydrocarbon chains of phospholipids near the area of the carbonyl groups of phospholipids of the lipid membrane [27]. The mentioned solvation of the membrane at 1.4 M DMSO correlated with decreased IC survival. This membrane effect of DMSO persisted even after DMSO removal.

DMSO can affect metabolic processes occurring in cells. It can cause membrane thinning and transmembrane pore formation [38–41]. In addition, it is osmotically active and may also cause unwanted cell excursions. The CPA decreased the indicators of IC survival. Apart from DMSO, D40, FBS and, to a lesser extent, HES did not lower the survival of ICs. In isosmotic conditions, PEG1500 and, especially, PEG400 showed damaging effects to ICs, supposedly, because of their additional osmotic activity and toxicity [42].

In our previous work [2], we have tested a number of common polymeric substances (common extracellular CPAs) for their ability to replace serum (or its components) in DMSO based cryoprotective solutions and found that the medium containing 0.7 M DMSO and 100 mg/ml D40 had an exceptional capability to preserve ICs. Other polymeric additives to the DMSO based media were less effective (HES) or non-effective (PEG1500 and PEG400). The indicators of IC survival in the medium supplemented with D40 were not inferior to the best serum-containing media with 1.4 M DMSO and 10% FBS. What's more, the D40 based media (also designated as 0.7DMSO+D40) did not contain components of serum. This is one more advantage of this medium because serum or its components can cause transmitting infection to biological material and lead to instability of composition of cryoprotective media from batch-to-batch [9-11]. The medium supplemented with D40 also had lower concentration of DMSO compared to its serum-containing counterpart, which, considering DMSO toxicity, is another advantage of the medium. Herein we have shown that D40, HES and PEG1500 mitigated the negative effect of DMSO on the general cell survival, probably due to their ability to prevent excessive cell volume fluctuations on cooling/warming. Although the metabolic activity of the

samples with 0.7 M DMSO, 0.7DMSO+D40 and 0.7DMSO+HES was the same, the indicator in 0.7DMSO+PEG1500 and 0.7DMSO+P400 was considerably lower. These indicate additional toxic effects of DMSO on cell components in the PEG-containing media.

0.7DMSO+D40 could suppress eutectic crystallization, promote the amorphous phase formation, inhibit intracellular crystallization, and change ice morphology in a way that promoted ICs survival. All the studied effects occur at a very wide temperature interval [2, 12, 13]. However, the contribution of osmotic effects of the tested cryoprotective solution developing on crystallization of water as well as their toxic effects were generally unexplored. Herein, we hypothesized that the additives could attenuate osmotic effects of the solution and DMSO toxicity. To verify this assumption, we investigated how the osmotic tolerance of ICs changed depending on the presence of DMSO, polymers and/or FBS. Additionally, we compared the data with cell survival obtained after cooling of the samples to -30 °C (the temperature interval from positive temperature to about -30 °C where osmotic and toxic effects of solutes are especially damaging [12, 37]) followed by warming and CPA removal.

DMSO presence exacerbated membrane damage caused by hypo- and hyperosmolarity. Absolute values of indicators of IC survival were generally lower in 1.4 M DMSO than in 0.7 M DMSO. However, in hypotonic media D40 was able to increase the OTL of ICs. Of note, PEG1500 was able to do this either. The extracellular CPAs can interact with the surface of cells preventing the membrane from excessive stretching [43]. Dextran in particular blocks membrane pores, thus, preventing cell death [44].

The polymers and FBS in the combined media 0.7DMSO+D40, 0.7DMSO+HES, 0.7DMSO+PEG1500, 1.4DMSO+FBS did not decrease the survival of ICs in hypotonic media compared to the media with the same DMSO concentrations without polymers and FBS. Moreover, the general survival of ICs in 0.7DMSO+D40 and 1.4DMSO+FBS tended to be higher than in 0.7 and 1.4 M DMSO, respectively. This means that D40 and FBS can attenuate the negative influence of DMSO on cell survival in hypo-osmotic conditions.

In hypertonic media, D40 and FBS, neither increase, nor decrease the OTL of ICs. All other additives caused lower indicators of cell survival (either general cell survival or metabolic activity preservation, or both). In hypertonic conditions, the use of 0.7 M DMSO did not lower metabolic activity. Furthermore, 0.7 M DMSO did not reduce any indicators of cell survival when used in combination with D40

The incubation of cells in hypo- or hyperosmotic conditions with DMSO or polymers, or FBS, is, to some extent, simulation of the events taking place during cryopreservation described at the beginning of Discussion. It is obvious, even if a substance can promote the survival of cells in hypo- or hyperosmotic solutions, it does not directly mean that it possesses adequate cryoprotective properties towards the cell. In our experimentation, for example, ICs can withstand hypo-osmotic conditions when PEG1500 was added to the solution. However, the additive was not able to promote amorphous phase formation or inhibit intracellular crystallization and recrystallization as effectively as 0.7DMSO+D40 [13]. PEG1500 was shown to be toxic towards ICs [42]. In the current research, in order to display the correlation between the survival of ICs in aniosmotic conditions and after cryopreservation clearer, ICs were cooled in cryoprotective media based on DMEM F12 only to -30 °C. The temperature interval (from positive temperature to -30 °C), where crystallization/melting of bulk water occurs, is responsible for most osmotic and toxic effects of cryoprotective media [14, 45, 46]. The cooling/warming to -30 °C has shown that 0.7DMSO+D40 was the most effective in terms of IC survival. Other combined media were less effective (0.7DMSO+HES, 1.4DMSO+FBS) or non-effective (0.7DMSO+PEG1500, 0.7DMSO+PEG400). Thus, D40 increases the survival of ICs, at least partially, at the expense of lowering osmotic effects of concentrated media and toxic DMSO effects. In our previous work serum-containing medium 1.4DMSO+FBS also

showed high cryoprotective properties, however, cooling to -30 °C resulted in moderate indicators of cell survival compared to other studied compositions. Apparently, this result related to high DMSO concentration in this composition, which could suppress salt eutectic, promote the amorphous phase formation, but could alter the membrane of ICs. This emphasizes the importance of finding approaches that lower DMSO concentration to the minimum required for effective cryopreservation. Cooling to -30 °C is not suitable for long-term preservation of cell samples as it does not prevent other physical transformations in the samples and in the present work was used to reveal the described effects associated with osmolarity changes. Lower temperatures are typically needed for cryopreservation of ICs and other cell suspension [2].

CONCLUSION

In the present research, we have shown that at 1.4M DMSO, the decrease in ICs survival correlated with the detected increase in the lipid membrane solvation with this solvent. The non-toxic additive D40 increased the osmotic tolerance limit of ICs in the hypo-osmotic medium and decreased the negative effects of DMSO on the cells. These effects were accompanied by high indicators of ICs survival obtained after cooling ICs to -30 °C with 0.7DMSO+D40. These results unveil the mechanisms of cryoprotection of 0.7DMSO+D40 and partially explain the superiority of 0.7DMSO+D40 media shown in our previous works compared with other investigated media. Understanding the mechanisms of cryodamage and cryoprotection of 0.7DMSO+D40 paves a way toward the development of new serum-/xeno-free cryoprotective compositions and improvement of cryopreservation protocols for cell suspensions that include many types of cells. Further studies are required to reveal the effects of DMSO on membranes and intracellular metabolic processes. The effects of other procedures of cryopreservation such as cooling/warming rates or controlled nucleation on the survival of ICs in 0.7DMSO+D40 has yet to be investigated.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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ВПЛИВ КРІОПРОТЕКТОРІВ НА ОСМОТИЧНУ ТОЛЕРАНТНІСТЬ ІНТЕРСТИЦІАЛЬНИХ КЛІТИН ЯЄЧКА

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Актуальність. Кріозбереження — це багатокроковий процес, який включає етапи, що механічно, осмотично та токсично впливають на біологічний матеріал. Використання кріозбереження біологічних матеріалів є економічно вигідним та забезпечує довготривале зберігання за кріогенних температур. Воно також гарантує стабільність генетичного компоненту клітин та знижує ризик контамінації біологічного матеріалу.

Мета роботи. Метою дослідження є вивчення впливу кріопротекторів (диметилсульфоксид (DMSO), декстран (D40), гідроксіетилкрохмаль, поліетиленгліколі (PEG1500 та PEG400) та фетальна бича сироватка) і їх комбінацій на взаємозв'язок між осмотичною толерантністю інтерстиціальних клітин яєчка (ІК) та кріозахистом.

Матеріали і методи. Поріг осмотичної толерантності (OTL) ІК та токсичний вплив кріопротекторів досліджували в середовищах на основі фосфатного буферного розчину з різною осмолярністю: ізоосмотичний (300 мОсм), гіпоосмотичний (225 мОсм) та гіперосмотичний (600 мОсм). Подібні осмотичні умови можуть розвиватися під час кріозбереження клітин у температурному діапазоні від +4 до -30 °C.

Результати. Показники виживання клітин після інкубації в середовищах залежали від осмолярності середовищ інкубації. Вони порівнювалися з показниками, отриманими після охолодження ІК до -30 °C з наступним нагріванням та видаленням кріопротекторів. Ми показали, що нетоксична добавка D40 підвищувала OTL ІК у гіпоосмотичному середовищі та зменшувала негативний вплив DMSO на клітини. Ці ефекти супроводжувалися високими показниками виживання ІК, отриманими після охолодження ІК до -30 °C у середовищі з 100 мг/мл D40 і 0,7 М DMSO.

Висновки. Ці результати розкривають механізми кріозахисту середовища 0.7DMSO+D40 і частково пояснюють перевагу цього середовища, яка була продемонстрована в наших попередніх роботах порівняно з іншими дослідженими середовищами. Розуміння механізмів кріопошкодження та кріозахисту 0.7DMSO+D40 відкриває шлях до розробки нових кріопротекторних композицій, вільних від сироватки та ксеногенних компонентів, а також покращення протоколів кріозбереження для клітинних суспензій, що включають багато типів клітин. Подальші дослідження необхідні для вивчення ефектів DMSO на мембрани та внутрішньоклітинні метаболічні процеси.

КЛЮЧОВІ СЛОВА: осмотична толерантність; токсичність; диметилсульфоксид; декстран; гідроксіетилкрохмаль; поліетиленгліколь; механізми кріозахисту.