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MODELING CELL OSMOTIC BEHAVIOR FOR IMPROVEMENT OF CRYOPRESERVATION PROTOCOL

Cryopreservation of cell suspensions, such as testicular interstitial cells (ICs), which include cells differing in size, volume, and membrane composition, is a very challenging task. Cryobiology requires the determination of several parameters to optimize the cryopreservation procedure, such as membrane water permeability (or hydraulic conductivity) (Lp) and its activation energy (Ea0), the permeability of the cell plasma membrane to dimethyl sulfoxide (DMSO) (Ps) and its activation energy (Ea1), and the osmotically inactive cell volume. This study aims to measure these key cryopreservation parameters for ICs. Additionally, it seeks to evaluate how quickly ICs become saturated with DMSO and to determine the optimal exposure time to cryoprotective media before cooling. By analyzing cell volume changes in different DMSO-based solutions, the research intends to optimize the cryopreservation process, potentially eliminating unnecessary exposure steps while maintaining cell viability. The kinetics of ICs volume changes in cryoprotective media with different DMSO concentrations (0.7—2.8 M), which additionally included 100 mg/ml dextran 40 (0.7DMSO + D40) or 10% FBS (1.4DMSO + FBS), were analyzed using the modified Kedem–Katchalsky model to determine the membrane transport properties. The calculated parameters clearly showed that cell saturation with DMSO occurred within a few minutes after its addition and could reach equilibrium before the estimated start of water crystallization in the samples. Thus, additional exposure of cells to DMSO-containing media was unnecessary. It could potentially lower the metabolic activity of ICs. Accordingly, the cryopreservation procedure for ICs can be shortened by removing the exposure step without reducing viability of ICs in 0.7DMSO + D40 and 1.4DMSO + FBS.

Key words: *serum-free media, xeno-free media, dextran, penentration coefficient, activation energy, dimethyl sulfoxide.*

INTRODUCTION

Cryopreservation of cells and tissues is a biotechnology that enables the long-term storage of biological material. In accordance with the classical two factor theory, cells can be damaged by growing ice crystals and by the highly concentrated solution that forms upon ice crystallization during cryopreservation [19]. Additionally, they may be affected by imbalanced tonicity upon the addition of cryopreservation media containing cryoprotective agents (CPAs) and during their removal after cryopreservation. Furthermore, the CPAs themselves can be toxic and cause various types of cellular damage. DMSO, one of the most effective CPAs, has been shown to exert both osmotic and toxic effects [29]. Cells may shrink upon DMSO addition due to water loss driven by the osmotic gradient, and then gradually regain volume as DMSO penetrates the cells. Upon DMSO removal, cells may expand beyond their osmotic tolerance limit, po-

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tentially causing damage [29]. DMSO can also induce membrane thinning, membrane dehydration, transmembrane pore formation, and disruption of internal cellular processes [9]. Therefore, optimal exposition time of testicular interstitial cells (ICs) with the media supplemented with CPAs can guarantee adequate cell saturation with the CPAs and minimized harmful toxic and osmotic effects of some CPAs.

In our previous research [21], we investigated different types of cryoprotective media containing DMSO for the cryopreservation of ICs. The media may additionally include some extracellular impermeant CPAs, such as dextran (D40), hydroxyethyl starch, polyethylene glycol, and other polymers. Alternatively, they may include fetal bovine serum (FBS). Serum-free medium containing 0.7 M DMSO and 100 mg/ml D40 (0.7DMSO+D40), and serum-containing medium supplemented with 1.4 M DMSO and 10% FBS (1.4DMSO + FBS), demonstrated the most effective cryoprotective properties for ICs. Their mechanisms of cryoprotection during cooling were somewhat different [21, 22]. However, their effects on cells before and after cooling remain under-investigated. One of the aspects, that can additionally improve the cryopreservation outcome in the above-mentioned DMSO-containing media is the optimal exposure time of cells with the media.

As for ICs, they include various cell types, such as Leydig cells, immune cells, peritubular cells, and others. Developing a cryopreservation protocol for such a heterogeneous cell suspension involves optimizing all steps of the cryopreservation process, which in turn must meet the requirements of most cells in the suspension. However, ICs differ in size, volume, and other characteristics, which makes the protocol development challenging. Plus, any parameter, could be critical for the survival of cells in the heterogenous suspension.

Creating an optimal cryopreservation protocol requires a combination of empirical testing of different cryoprotective compositions, cooling rates, and CPA addition/removal strategies, as well as modeling the general behavior of cells during cryopreservation. Membrane permeability is one of the key factors determining the cell osmotic behavior in cryoprotective media during cryopreservation. The likelihoods of damaging intracellular ice formation or cell injury caused by the hyperconcentrated solution surrounding the cells are largely predetermined by membrane permeability to water and CPAs [30, 33]. Membrane permeability is characterized by the permeation coefficients for water (L_p) and a permeating CPA (P_s), such as DMSO, as well as the corresponding activation energies for their transport (E_{a0} and E_{a1}). Therefore, the objective of this research is to determine the average values of L_p, P_s, E_{a0}, and E_{a1}; assess the degree of cell saturation with DMSO; and justify the optimal exposure time of ICs in the cryopreservation media prior to cooling.

MATERIALS AND METHODS

Experimental animals. Male rats from the animal house (aged 5—6 months, 300—400 g) of the Institute for Problems of Cryobiology and Cryomedicine of the NAS of Ukraine (Kharkiv) were used. The animals were provided with standard laboratory chow and water *ad libitum*. All manipulations with animals were approved by the Bioethics Committee of the Institute for Problems of Cryobiology and Cryomedicine of the NAS of Ukraine (Protocol No. 4, dated of June 16th, 2025) and comply with the main provisions of the 'European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes' (Strasbourg, 1986) and the Law of Ukraine 'On the Protection of Animals Against Cruelty'.

Isolation of testicular interstitial cells. Cervical dislocation was used for scarification of the rats. The bodies of animals were immersed in 75% ethanol for 5 minutes. The testes were isolated, decapsulated, and the blood vessels were trimmed off the testes. Then, they were placed in 15-mL centrifuge tubes containing 4 mL of DMEM F12 per testis, supplemented with enzymes: 0.2 mg/mL collagenase (type I) (Sigma-Aldrich, USA) and 0.1 mg/mL DNase I (Sigma-Aldrich, USA) for 10 minutes. The tubes were placed in a thermostated shaking water bath at 90 cycles/min and 34 °C. Then, 10 mL of collagenase-free DMEM F12 (Biowest, France) was added to each tube. After that, the seminiferous tubule mass was removed by filtration through double 100-μm-nylon mesh. The filtrates were centrifuged at 325 g for 3 minutes at room temperature. The supernatants were discarded, and the residues were resuspended in 10 mL of phosphate-buffered saline (PBS). The sedimentation procedure was repeated. The cell concentration was adjusted to 4×10^7 cells per mL with PBS. Then, the cell suspensions were diluted with concentrated cryopro-

tective media (1 : 1) to reach the final concentration of additives (see below). Thus, the final concentration of cells in the sample was 2×10^7 cells/mL.

Osmotic behavior of ICs. Cells were exposed to different osmotic solutions of PBS (300 mOsm (isosmotic), 600, 900, and 1200 mOsm) by mixing the obtained cell suspension with a stock solution of ×10 concentrated PBS and distilled water. The ICs shrank in hypertonic solutions due to water efflux. The equilibrium volumes of ICs were determined microscopically. The Boyle–Van't Hoff relationships for both groups were established by plotting cell volumes at different PBS concentrations against the reciprocal of the corresponding osmolarities. The osmotically inactive volume of the cells, which includes solutes and osmotically inactive water, was estimated by extrapolating the Boyle–Van't Hoff plot to the y-axis.

Determination of permeability coefficients and activation energies. The measurement of permeability coefficients for water (L_p) (hydraulic conductivity) and DMSO (P_s) (DMSO permeability) was carried out by tracking the kinetic changes in cell volume when cells were exposed to anisotonic environments. This was achieved by adding equal volumes of PBS or solutions containing cryoprotective agents to the cell suspension, thereby reaching the target final concentrations of these substances in the medium. Data were obtained using an AxioObserver Z1 microscope (Carl Zeiss, Germany) equipped with a ×10 objective lens, a camera for image acquisition, and a temperature-controlled chamber to stabilize and maintain the desired sample temperature. Images were analyzed using the image processing software AxioVision Rel.4.8 (Carl Zeiss, Germany). Cell images were captured at 10-second intervals, and changes in cell volume were monitored over 600 seconds. Since the cells remained approximately spherical throughout the observation period, the average cell diameter was calculated by measuring and averaging several diameters (at least three) taken at different angles. Subsequently, the change in cell volume over time was presented as a normalized volume, defined as the ratio of the cell volume at a given time to the initial volume.

Experimental curves showing the change in final cell volume over time were constructed as follows: first, the experimental data were compared with theoretical predictions. Then, the root mean square deviations were calculated by subtracting the values of the theoretical curve from the experimentally obtained normalized volumes. These differences were squared, summed, and the minimum sum of squared deviations was determined. The theoretical curve with the lowest root mean square deviation — based on a nonlinear equation and the Kedem–Katchalsky model [12], modified by E.A. Gordienko and M.S. Pushkar, and adapted for a single cell [7, 27] — was used to determine the permeability coefficients for water and DMSO.

The activation energy can be calculated using the slope of the Arrhenius plot of $Ln(L_p)$ (or $Ln(P_s)$) versus 1/T: E_{a0} (or E_{a1}) = $-1 \times$ (Slope $\times R_0$), or by using the equation:

$$E_{a0} = -(8.314 \times T_0)/((1-(T/T_0)) \times Ln(L_pT/L_pT_0)$$
$$E_{a1} = -(8.314 \times T_0)/((1-(T/T_0)) \times Ln(P_sT/P_sT_0),$$

where E_{a0} and E_{a1} are activation energies for water and DMSO transport respectively. It should be noted that the results obtained using the two methods did not differ significantly; however, the first method provides a more integrative indicator, as it includes measurements at 20, 15, 10, and 4 °C. A significant deviation from the linear trend may indicate possible phase transitions. The second method is more convenient for calculating activation energies between two specific temperature points.

The kinetics of changes in concentration of the solution surrounding the cells during the crystallization was modeled by approximating the phase diagram of the freezing point of aqueous DMSO solutions, as described by A.F. Todrin et al. [28]. One clarification should be made: the term "intracellular osmotically active substances" refers to the osmotically active intracellular content excluding CPAs. This term is expressed in mOsm. However, the intracellular content includes substances whose behavior in aqueous solutions deviates from ideality, such as polymers (*e. g.*, proteins). Expressing osmotic behavior in mOsm means that the osmotic pressure generated by the intracellular osmotically active content is equivalent to the osmotic pressure of a NaCl solution of the same osmolarity in water.

Measurements of indices of cell survival. For measuring cell survival indices, ICs were mixed with 0.4% Trypan Blue dye (1 : 1) and counted in a hemacytometer chamber. General cell survival was determined as the ratio of the total number of cells in a sample after incubation to the number of cells in PBS (300 mOsm) before incubation, multiplied by 100%. Cells that remained unstained by Trypan Blue dye were considered to have intact mem-

branes. The survival of cells with intact membranes was calculated as the ratio of the number of unstained cells in a sample after incubation to the number of unstained cells in PBS (300 mOsm) before incubation, multiplied by 100%.

To measure the metabolic activity of ICs after incubation, 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 following incubation at 4 °C and removal of the medium. The cells were incubated with MTT for 2 h at 35 °C. After incubation, ICs were sedimented at 325 g for 3 min, and the supernatant was discarded. Then, 450 μL of DMSO was added to the stained pellet, and the samples were stirred. After 5 minutes, the samples were centrifuged, and the optical density (extinction) of the supernatant was measured at 530 nm. Samples containing no cells were used as a reference. Metabolic activity preservation was calculated as the ratio of the optical density of a sample after incubation to the optical density of a sample in PBS (300 mOsm) before incubation, multiplied by 100%.

Histochemical staining for 3β-hydroxysteroid dehydrogenase (3β-HSD) activity was performed to identify 3β-HSD$^+$ cells in the samples. The procedure was carried out according to the method described by G.R. Klinefelter *et al.* for the determination of Leydig cells in a suspension of testis-derived ICs [14]. In brief, 50 μL of ICs suspension was placed onto slides and incubated at room temperature for 1 hour to allow the cells to dry. Subsequently, the slides were covered and stained with a mixture of solutions A and B. Solution A consisted of 1 mg nitro-blue tetrazolium (Sigma) and 0.6 mg dehydroepiandrosterone (Sigma) dissolved in 1 mL DMSO, while Solution B contained 10 mg β-NAD (Sigma) dissolved in 9.5 mL PBS. Staining was carried out for 90 minutes. After staining, the cells were rinsed with distilled water and fixed in PBS supplemented with 10% formalin and 5% sucrose. The slides were then mounted with a PBS:glycerol solution (1 : 1) and covered with cover slips. The 3β-HSD$^+$ cells, positive for 3β-hydroxysteroid dehydrogenase activity, were identified by the presence of dark blue formazan granules. The 3β-HSD$^+$ cell survival was calculated as the ratio of the number of 3β-HSD$^+$ cells after cryopreservation to the number before cryopreservation, multiplied by 100%.

Cryopreservation. To achieve the final DMSO concentration, 0.5 mL of ICs (4×10^7 cells/mL) were diluted (1 : 1) with concentrated cryoprotective media (4 °C) in 1.8 mL cryocontainers (Nunc, Denmark). The samples were cooled in a programmable freezer at a rate of 1 °C/min to −80 °C, then plunged into liquid nitrogen (−196 °C). The samples were warmed in a water bath at 37 °C until the crystal phase disappeared. The cryoprotective media were removed by gradually diluting the samples with Ham's F12 medium. The cell suspension was centrifuged, and the supernatant was discarded. The remaining ICs were resuspended in Ham's F12 medium, and the procedure for cryoprotective medium removal was repeated. Finally, the volume of the IC suspension was adjusted to 1 mL with Ham's F12, and indicators of cell survival were measured.

Statistical analysis. All data are presented as medians (25^{th} percentile; 75^{th} percentile) throughout the text. The data were assessed using the Mann–Whitney U-test with Bonferroni correction. The determination coefficient (R^2) was used to describe how well the lines fit data points. All quantitative data were processed with STATISTICA, version 10 (StatSoft Inc., USA).

RESULTS

Determining osmotically inactive volume. The ICs are a cell suspension containing various cell types. It has been shown that among them, one specific type is microscopically distinguishable from the others due to its noticeably larger volume [22]. The cells come to 5—7% of all ICs. The diameters of these large cells range from approximately 18.3 to 19.8 μm. For clarity, they are referred to as "large cells" (L-ICs), in contrast to the remaining cells within the ICs, which are designated as "small cells"(S-ICs). The mean osmotic parameters, particularly in the osmotically inactive volumes, of L- and S-ICs differed (Fig. 1). The fitted line R^2 coefficients of L-ICs are higher (0.9737) than those of S-ICs (0.8435), which reflects the higher heterogeneity of S-ICs.

Osmotic behavior of ICs in the solution of permeating and nonpermeating solutes. In the hypertonic PBS (600 mOsm) solution, the volume of ICs decreased and reached its final value (Fig. 2, *a*). The initial volume of IC-L was reduced by 40%. The volume change was temperature-dependent: the higher the temperature, the faster the volume loss. At 4 °C, the new stable volume was reached in

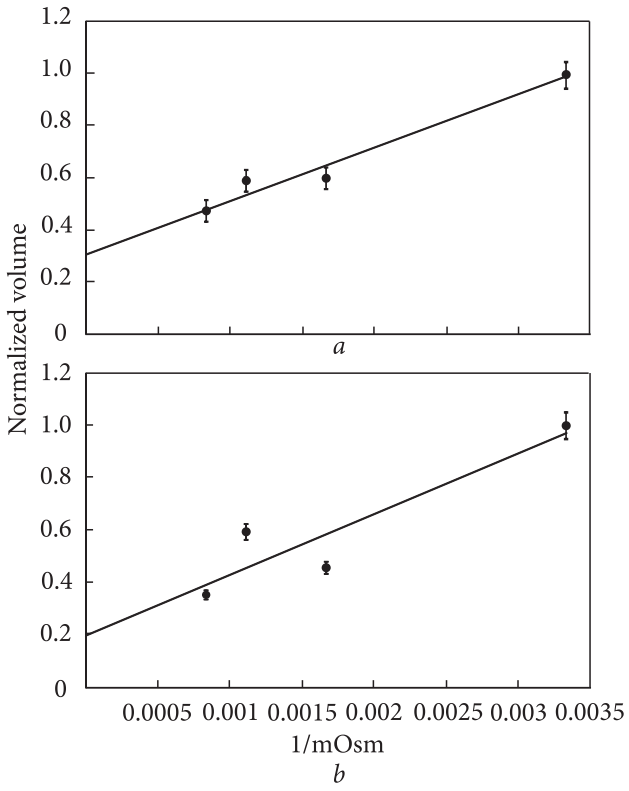

Fig. 1. Boyle Van't-Hoff plot for L-ICs (*a*) and S-ICs (*b*). These linear regressions were fitted for hypertonic data points obtained in PBS of differing osmolarities. Values are represented as mean and SD. Crossings with y-axis show osmotically inactive volume

3 minutes, whereas at 20 °C, the main changes occurred within a minute. The osmotic behavior of S-ICs was similar; however, in the PBS (600 mOsm), the cells experienced a greater volume loss (up to 50%) (data not shown). Since the components of PBS do not penetrate the membrane under the studied conditions, the cell volumes remained reduced.

Conversely, in isosmotic PBS supplemented with the permeating solute of 0.7 M DMSO, cell volumetric changes exhibited a descending and ascending phase. The initial descending phase corresponded to the cell volume loss largely due to water efflux, while the ascending phase was caused by DMSO entry into the cells, accompanied by the restoration of cell volume. The dynamics of volume changes also depended on temperature. In media with higher DMSO (1.4 M) concentrations, the downward protrusion of the curve was more pronounced (Fig. 2, *b*) due to a higher degree of dehydration. Volume changes in S-ICs were similar (data not shown). It can be observed that in DMSO solutions, the volume of ICs did not fully return to its initial value. This observation can be considered a deviation from the Kedem-Katchalsky formalism [12], which predicts complete restoration of cell volume (and, in some cases, when the media is supplemented with a permeant CPA, even an increase in cell volume) [18].

In the combined medium supplemented with 0.7 M DMSO and 100 mg/ml D40 (0.7DMSO + + D40), ICs experienced a higher volume loss. In fact, both L-ICs and S-ICs lost about half of their volume at 4 °C during the first minute (Fig. 2, *c*). Subsequently, the cell volume increased due to the entry of DMSO into the ICs.

However, the restoration of cell volume was partial. Even after 10 minutes of observation, the final volume of L-ICs at 4 °C was approximately 25% lower than the initial volume. The volume of S-ICs remained reduced by almost 40% after 10 minutes (data not shown). In the combined medium supplemented with 1.4 M DMSO and 10% FBS (Fig. , *d*), the cell volume changes were similar to those in the medium containing 1.4 M DMSO only. After 10 minutes of incubation, the final volume of both L-ICs and S-ICs at 4 °C was approximately 10% lower than the initial volume.

Determining coefficients of permeability for water (L_p) and DMSO (P_s). The hydraulic conductivity and permeability of the cell plasma membrane to DMSO for all media were lower at 4 °C (Table 1). DMSO significantly decreased the L_p of both L- and S-ICs compared with the L_p measured in hyperosmotic PBS (600 mOsm). This effect was dose-dependent and was observed in both L- and S-ICs.

In media with DMSO concentrations ranging from 0.7 to 2.1 M the membrane permeability to DMSO of L-ICs was relatively stable (Table 1). The value of P_s for L-ICs in 2.8 M DMSO at 4 °C was significantly lower compared with other DMSO concentrations ($p < 0.05$). However, at 20 °C, the opposite effect was observed: at low temperature (4 °C), DMSO movement across the plasma membrane decreased, while at higher temperatures (20 °C), 2.8 M DMSO had a detrimental effect on the plasma membrane which increased its permeability to DMSO. This may involve DMSO interaction with membrane phospholipids, reducing membrane thickness [10], or promoting the formation of pores [6].

Plasma membranes of L- and S-ICs in the 0.7DMSO + D40 medium had higher L_p and lower P_s compared to the media supplemented with0.7 M DMSO. The addition of 10% FBS

(1.4DMSO + FBS) had slight (if any) effect on L_p and P_s of L-ICs compared to the medium containing 1.4 M DMSO only. As for P_s of S-ICs, the values showed no clear trends at 4 °C, although at 2.1 M DMSO, it was higher than in the media with other DMSO concentrations. This might be due to the general heterogeneity of S-ICs. In 2.1 M, and especially in 2.8 M DMSO, P_s of S-ICs significantly increased ($p < 0.05$).

The values of E_{a0} for water transfer indicate that water crosses the membrane through facilitated diffusion via specialized channels, while relatively higher E_{a1} values suggest that DMSO moves across the lipid bilayer by simple diffusion under the given conditions [13]. DMSO significantly increased E_{a0} for both groups of ICs, implying that the water transfer across the plasma membrane was inhibited by DMSO (Table 2). It influences the interactions between phospholipid head-groups, leading to an increase in the lateral expansion of the lipid bilayer [9]. This effect may result from membrane dehydration and changes in the lipid order of the membrane [23]. These alterations could explain the observed changes in water transport in DMSO solutions.

The activation energy for water transfer measured for L-ICs was reduced in the 0.7DMSO + + D40 and 1.4DMSO + FBS media compared with the medium containing the corresponding concentrations of DMSO (Table 2). In contrast, the indicator increased in S-ICs. The combined medium of 0.7DMSO + D40 lowered E_{a1}, which would suggest an enhancement of DMSO transfer into ICs, when other factors such as slowed and/or partial cell volume recovery are excluded. The addition of 10% FBS (1.4DMSO + FBS) had minimal effect on DMSO transfer across the membrane when compared to 1.4 M DMSO alone.

As improving the cryopreservation outcome for ICs can be achieved by either increasing DMSO concentration from 0.7 to 1.4 M or by adding D40 or FBS, further research was focused on examining the differences among these media. The methods used to determine activation energies of molecular transport do not account for potential sharp changes in L_p and P_s (and consequently the corresponding activation energy) caused by phase transitions in membranes or significant alterations in the physical and chemical properties of the membrane under the influence of substances such as DMSO, which can markedly modify their physical and chemical characteristics. Assuming the absence of such transitions (or their negligible impact), the dependence of $Ln(L_p)$ or $Ln(P_s)$ on 1/T (where T is the absolute temperature) should follow a linear trend.

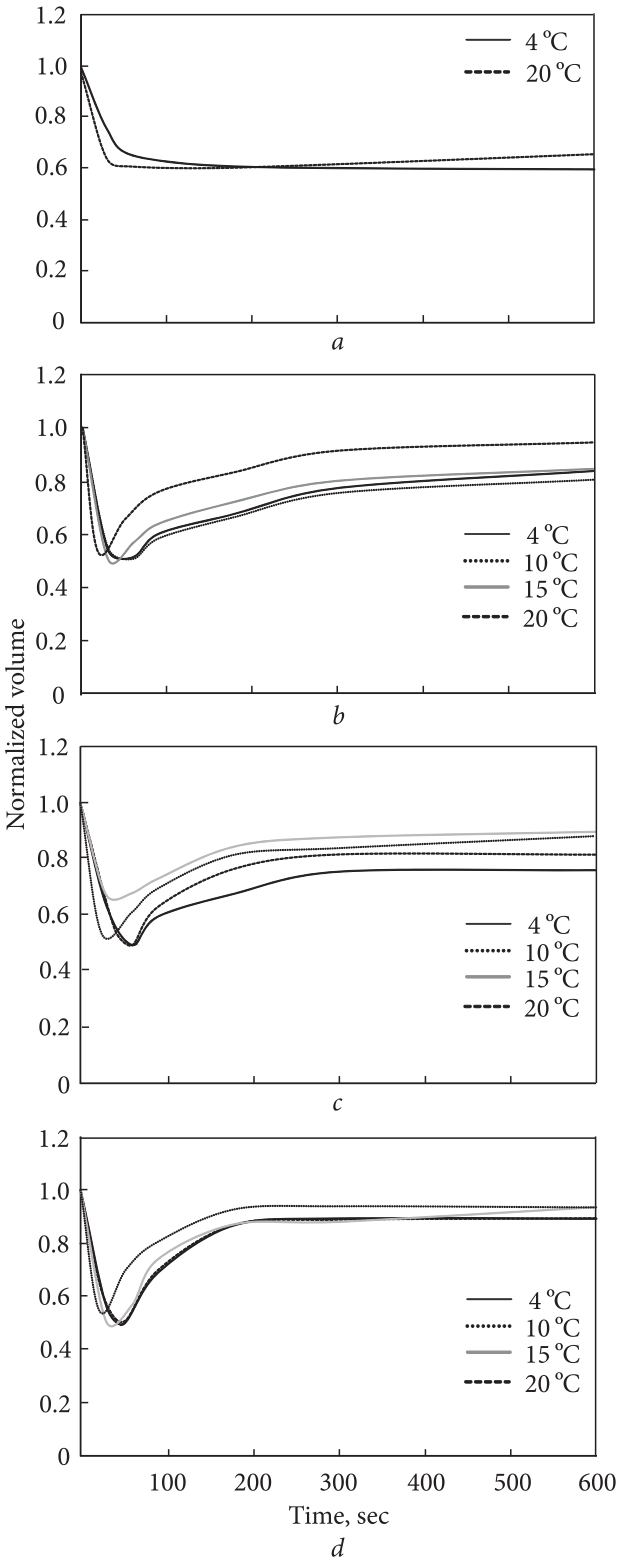

Fig. 2. Dynamic of L-ICs volume changes (*a*) in hypertonic solution of PBS (600 mOsm), (*b*) 1.4 M DMSO Ham's F12 (300 mOsm), (*c*) 0.7DMSO + D40, (*d*) 1.4DMSO + + FBS

However, the observed deviations from linearity (Fig. 3) suggest that DMSO and additives induce some physical and chemical changes in the membrane, implying potential deviations from classical Kedem-Kachalsky formalism.

The lines (their slope and intercept with the y-axis) and the points for 0.7 and 1.4 M DMSO were almost identical and showed similar behavior to the 1.4DMSO + FBS solution (Fig. 3). This suggests that, based on the indices of membrane permeability, the changes in the membrane induced by 0.7 and 1.4 M DMSO were generally the same for both L-ICs and S-ICs in these three solutions. However, the locations of points and lines for the 0.7DMSO + D40 medium were distinctly different from all the aforementioned solutions. This indicates a unique effect of D40 on the transport of DMSO and water across the plasma membrane.

Using the Kedem-Kachalsky formalism to determine cell saturation with DMSO and predict the du-

Table 1. **Hydraulic conductivity and permeability coefficient of the cell plasma membrane to DMSO for testicular interstitial cells at 4 and 20 °C (median (25^{th} and 75^{th} percentiles))**

L-ICs	Lp, (m/Pa/sec) × 10^{-14}		Ps, (m/sec) × 10^{-8}	
	4 °C	20 °C	4 °C	20 °C
PBS (600 mOsm)	5.0 (3.5; 7.9)	8.5 (7.3; 9.4)	—	—
0.7 M DMSO	1.4 (1.2; 1.7)	4.0 (3.1; 4.9)	2.1 (1.9; 2.4)	10.6 (7.8; 12.6)
1.4 M DMSO	1.8 (1.3; 2.3)	4.4 (3.9; 5.2)	2.3 (1.7; 2.8)	10.6 (7.7; 13.0)
2.1 M DMSO	1.0 (0.8; 1,5)	2.3 (1.9; 3.0)	2.4 (1.8; 3.2)	10.6 (8.1; 12.5)
2.8 M DMSO	0.6 (0.1; 1.0)	1.3 (0.9; 2.0)	0.6 (0.2; 1.1)	21.1 (15.6; 23.5)
0.7DMSO + D40	2.7 (1.9; 3.2) *	5.7 (4.8; 6.6) *	1.1 (0.5; 1.4) *	3.2 (2.4; 3.9) *
1.4DMSO + FBS	1.8 (1.2; 2.5) #	4.0 (3.4; 4.9) #	2.5 (1.9; 3.3) #	12.9 (10.4; 15.5) #

S-ICs	Lp, (m/Pa/sec) × 10^{-14}		Ps, (m/sec) × 10^{-8}	
	4 °C	20 °C	4 °C	20 °C
PBS (600 mOsm)	6.6 (5.2; 8.1)	8.8 (6.2; 11.5)	—	—
0.7 M DMSO	0.9 (0.4; 1.4)	1.7 (1.2; 2.3)	2.1 (1.4; 3.0)	10.4 (7.3; 11.9)
1.4 M DMSO	0.9 (0.4; 1.4)	1.5 (0.9; 2.2)	2.2 (1.3; 3.1)	11.6 (8.2; 12.1)
2.1 M DMSO	0.9 (0.3; 1.5)	1.3 (0.6; 2.3)	5.2 (3.3; 6.3)	69.4 (30.3; 82.0)
2.8 M DMSO	0.7 (0.2; 1.2)	1.1 (0.6; 1.9)	2.1 (1.2; 3.0)	208.1 (103.3; 250.4)
0.7DMSO + D40	1.6 (0.9; 2.2) *	3.8 (2.5; 4.6) *	0.8 (0.3; 1.2) *	1.7 (0.9; 2.3) *
1.4DMSO + FBS	1.3 (0.7; 2.0) *	2.6 (1.5; 3.2) *, #	2.2 (1.5; 3.1) #	9.9 (8.1; 11.7) #

Notes: L_p at 20 °C were statistically higher than at 4 °C, $p < 0.05$; all media had statistically lower L_p compared with PBS (600 mOsm), $p < 0.05$; * the values were statistically different from the medium having the same DMSO, $p < 0.05$; # the values were statistically different from 0.7DMSO + D40, $p < 0.05$.

Table 2. **Activation energies for water and DMSO transfer across the plasma membrane for testicular interstitial cells at 4 and 20 °C (median (25^{th} and 75^{th} percentiles)), kJ/mol**

Solutions	L-ICs		S-ICs	
	Ea0 (water), kJ/mol	Ea1 (DMSO), kJ/mol	Ea0 (water), kJ/mol	Ea1 (DMSO), kJ/mol
PBS (600 mOsm)	21.2 (11.4; 29.3)	—	11.5 (7.0; 12.8)	—
0.7 M DMSO	37.9 (37.4; 41.9)	64.6 (56.4; 66.2)	26.3 (25.4; 39.1)	63.9 (56.2; 64.2)
1.4 M DMSO	35.7 (32.6; 43.8)	61.0 (60.3; 61.3)	20.4 (17.6; 32.4)	66.4 (55.3; 73.5)
2.1 M DMSO	33.2 (27.7; 34.5)	59.3 (54.4; 60.0)	14.7 (11.5; 17.1)	102.4 (88.5; 103.4) *
2.8 M ДМСО	30.9 (27.7; 55.3) *	142.1 (122.2; 171.3) *	20.4 (18.3; 43.9)	181.1 (177.8; 183.4) *
0.7DMSO + D40	29.8 (28.9; 37.0) *	42.6 (40.9; 62.6) *	34.5 (31.8; 36.6) *	30.1 (26.0; 35.6) *
1.4DMSO + FBS	31.9 (26.9; 41.6) *	65.5 (61.7; 67.9)	26.4 (18.8; 27.7)	63.8 (60.0; 67.3)

Notes: E_{a0} of all media was statistically different from PBS (600 mOsm), $p < 0.05$; * the values were statistically different from 0.7 M DMSO, $p < 0.05$.

ration of the saturation process before cryopreservation. The assessed parameters of plasma membrane permeability can be used to predict the degree of cell saturation with DMSO depending on time, temperature, and the osmolarity of intracellular components of ICs [16, 17]. This, in turn, may scientifically justify the optimal time and temperature at which the ICs should be kept in cryoprotective media, which includes DMSO and other additives, before starting the cooling process.

The modeling predicts that at a temperature of 4 °C, the main volumetric changes of ICs end within 300—400 seconds. In 0.7 and 1.4 M DMSO media, 90% saturation of cells with DMSO is reached within 300—400 seconds (Fig. 4). Most changes occur within the first 90–120 seconds. The concentration of intracellular osmotically active substances reaches 400 mOsm in 300—400 seconds, which is 100 mOsm higher than in ICs placed in isotonic medium without additives. The concentration steadily tends to decrease over the remaining incubation period. Fluctuations in intracellular osmolarity are higher in 1.4 M DMSO and 1.4DMSO + + FBS due to the higher DMSO concentration, which, in turn, causes significant water efflux from ICs. The changes in L-ICs and S-ICs were generally similar. The model also predicts that in 0.7DMSO + D40, the saturation of ICs with DMSO would require an additional 50—100 seconds. The predicted fluctuation of intracellular osmolarity was also significant in this medium. Increased temperature resulted in faster changes (not shown).

Taking into account the estimated duration of cell volume excursions, the saturation of cells with DMSO, and the changes in the concentrations of osmotically active intracellular components, an adequate contact time of ICs with DMSO in cryopreservation media is approximately 4 to 7 minutes. However, this period is generally comparable to the time required for a sample (~1 ml) to cool from 4 to –2 °C in a programmable freezer chamber at a cooling rate of approximately 1 °C/min. Within this temperature range, the sample remains in a liquid state, as –2 °C is above the melting point of the medium containing 0.7 M DMSO [22]. Therefore, the effective contact time of ICs with DMSO during cooling may be even longer — since supercooling below the melting point can occur and takes additional time — despite the ICs being mixed with DMSO immediately prior to the start of cooling. Even in the 0.7DMSO + D40 medium, where volumetric and concentration changes were the slowest among the tested solutions, these estimations indicate that, in theory, ICs can be cooled immediately after being mixed with the cryoprotective medium.

Optimization of cryopreservation. The media with the highest cryoprotective properties showed almost identical results with and without a 30-minute exposure prior to cooling at a rate of 1 °C/min (Table 3). The only exception was the metabolic activity of ICs cryopreserved without exposure in 1.4DMSO + FBS; in this case, metabolic activity was higher without the exposure. These data demonstrate the potential to optimize the cryopreservation protocol by reducing incubation time. Unnecessary exposure of ICs to DMSO before and after cryopreservation can otherwise prolong the procedure and increase the risk of cell damage due to DMSO toxicity [1].

DISCUSSION

Studying the osmotic behavior of cells in the media supplemented with CPAs is critical for developing an optimal cryopreservation protocol. A typical cryopreservation protocol can be divided into several stages, including isolation of biological material, addition of cryoprotective medium containing CPAs, exposure of cells to the medium prior to cooling, samples' cooling, storage, warming, removal of the cryoprotective medium, transfer of cells to CPA-free medium, and assessment of cell recovery after cryopreservation. Most cryobiological studies focus on investigating the effects of various chemicals and cryoprotective compositions on cell survival during cryopreservation, as well as on cooling and warming rates; however, relatively little attention has been paid to the rationale behind the exposure time of cells to CPAs.

Some studies investigate exposure time empirically, with durations ranging from seconds to 30 minutes [11, 32]. Such a mechanistic approach can be effective. However, improving the cryopreservation procedure requires the development of specific methodologies and a knowledge base that describe the rate of CPA penetration into cells and the associated volumetric changes. One such methodology is the Kedem-Katchalsky formalism. In the present work, we demonstrated the feasibility of using a modified Kedem-Katchalsky formalism to predict the behavior of ICs, a heterogeneous cell suspension composed of different cell types. We

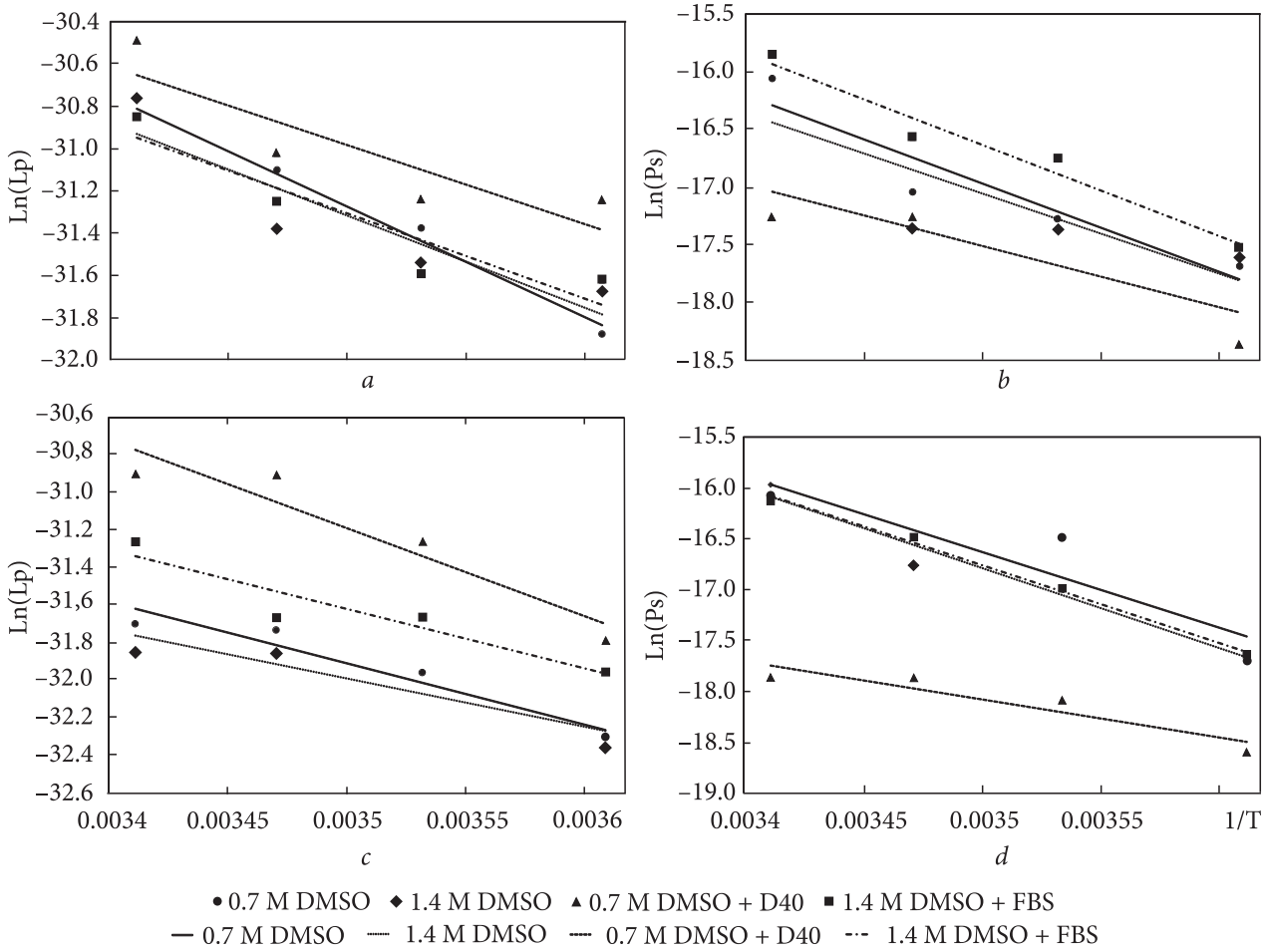

Fig. 3. Arrhenius relationships between the natural logarithm of L_p for L-ICs (*a*), P_s for L-ICs (*b*), L_p for S-ICs (*c*), P_s for S-ICs (*d*) and 1/T, where T is the absolute temperature (L_p and P_s were measured at 4, 10, 15, 20 °C)

also identified some deviations from the classical model that may affect the final outcome of cryopreservation, such as incomplete restoration of cell volume or potential membrane transformations caused by cryoprotectants and temperature changes.

The development of cryopreservation methods for heterogeneous suspensions involves specific challenges, particularly the need to select cryopreservation parameters that are suitable for the majority of cells in the suspension [2]. Therefore, even seemingly minor aspects of the cryopreservation process can play a significant role for certain cell types within a heterogeneous suspension. Cryopreservation of a heterogeneous suspension also necessitates obtaining averaged membrane permeability indicators [15]. For example, our modeling suggests the need to shorten the exposure time of ICs to DMSO before cooling. DMSO is a widely used cryoprotective agent, but it can exert osmotic and toxic effects on cells [20]. Consequently, excessive exposure may lead to unnecessary cell death, while insufficient exposure can result in suboptimal cryopreservation outcomes in terms of cell survival.

In our previous works, we demonstrated that certain cryopreservation compositions are beneficial for the preservation of ICs: the serum-free medium 0.7DMSO + D40 and the serum-containing medium 1.4DMSO + FBS [21]. These compositions were also the focus of the present study. Both contain either the impermeating agent D40 or a protein component of serum, which can potentially interfere with water and DMSO distribution across the membrane, resulting in incomplete volume recovery.

The modified Kedem-Katchalsky formalism indicated that the saturation of ICs with DMSO was completed within a few minutes, allowing for a reduction in exposure time. Typical cooling of a 1-ml sample in a cryocontainer at a set rate of 1 °C/min (such as the one demonstrated in the present work) requires at least 7 to 20 minutes before water

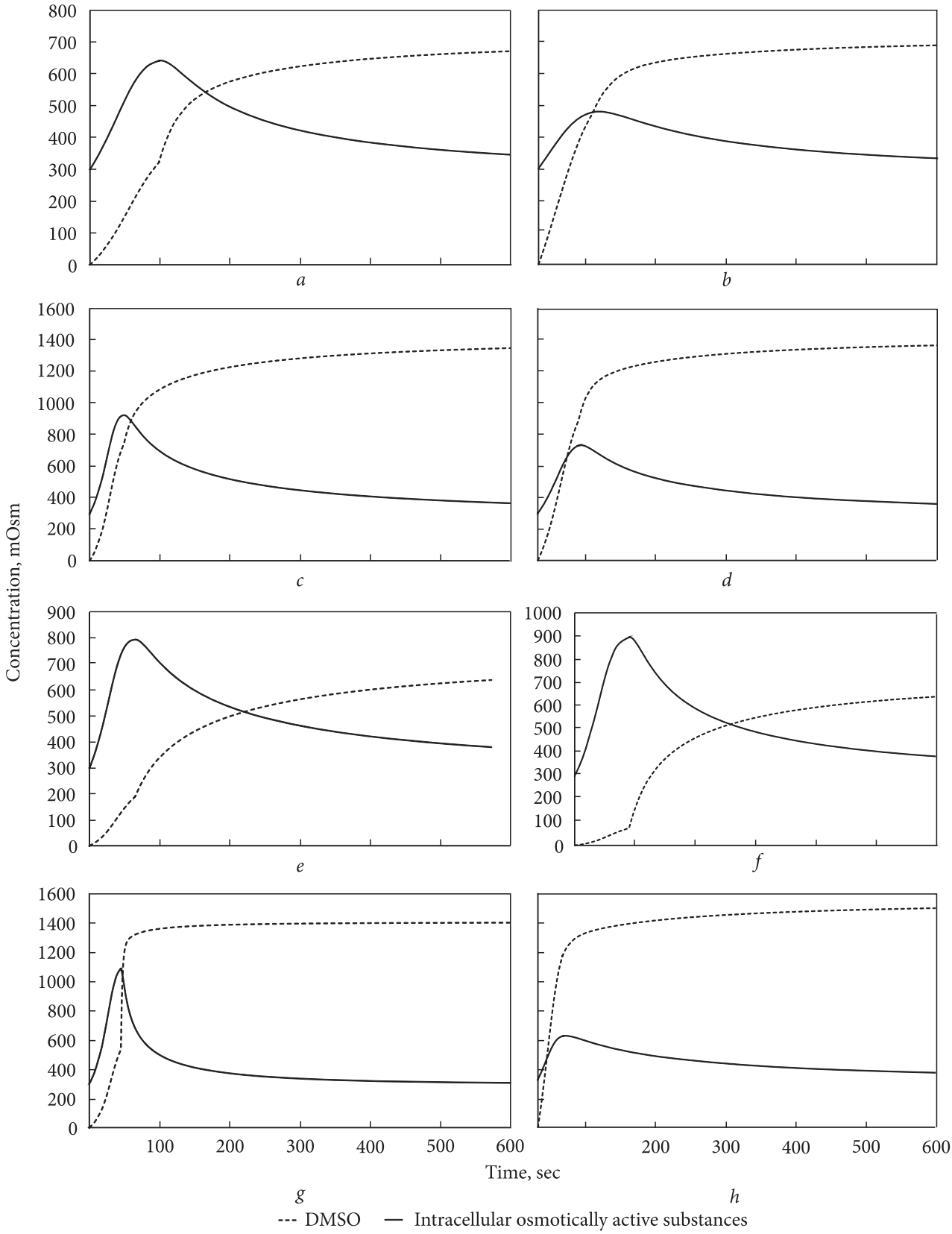

Fig. 4. Predicted changes of intracellular concentration of DMSO and osmolarity of intracellular components: *a* — L-ICs in 0.7 M DMSO; *b* — S-ICs in 0.7 M DMSO; *c* — L-ICs in 1.4 M DMSO; *d* — S-ICs in 1.4 M DMSO; *e* — L-ICs in 0.7DMSO + D40; *f* — S-ICs in 0.7DMSO + D40; *g* — L-ICs in 1.4DMSO + FBS; h — S-ICs in 1.4DMSO + FBS. Temperature: 4 °C

Table 3. **Indices of cell survival after cryopreservation**

Indices	0.7DMSO + D40		1.4DMSO + FBS	
	30 min exposure	without exposure	30 min exposure	without exposure
General cell survival, %	36 (32; 52)	36 (26; 54)	27 (26; 32)	31 (23; 39)
Plasma membrane preservation, %	36 (23; 41)	36 (22; 46)	21 (12; 25)	25 (10; 30)
Metabolic activity preservation, %	52 (50; 53)	52 (50; 53)	39 (37; 40)	45 (44; 46) *
3β-HSD$^+$ cell survival, %	58 (54; 63)	65 (56; 72)	24 (19; 34)	32 (26; 42)

Notes: * the values were statistically different from the sample that was exposed to cryoprotective media prior to cooling during 30 min, $p < 0.05$.

crystallization begins in the sample. Of course, this also depends on the starting temperature, the degree of supercooling, the cryoprotective composition, the material the cryocontainer is made of, its shape, and so forth. Therefore, ICs mixed with the cryoprotective composition do not require additional exposure time, as they come into contact with the medium immediately during cooling and have sufficient time for DMSO equilibration across the plasma membrane.

Empirical evidence supported this prediction, as cryopreservation with minimized exposure time yielded favorable outcomes. Moreover, the metabolic activity of ICs cryopreserved with 1.4DMSO + + FBS was even lower in the samples exposed to DMSO for 30 minutes, clearly demonstrating DMSO toxicity. In our previous study [23], we showed that high concentrations of DMSO can alter the plasma membrane. Therefore, using cryopreservation media with lower DMSO concentrations can reduce the risks associated with DMSO toxicity.

The 0.7DMSO + D40 medium is a suitable example, as it lacks serum components that may promote infections and fail to ensure batch-to-batch consistency of cryoprotective media [3, 6, 8]. In this study, based on the assessed membrane permeability parameters, we demonstrated a potential avenue for optimizing cryopreservation protocols for ICs by shortening the exposure time to DMSO-containing media. This medium aslo promoted the survival of some specific differentiated cell such as 3β-HSD$^+$ cell, although their functionality in terms of androgen secretion has yet to be clarified. Some studies have shown that dextrans can interact with cellular surfaces, though this interaction does not lead to significant dehydration [20, 23]. The association of polysaccharides with cellular membranes may help retain water near the lipid bilayer surface [26, 31]. Certain CPAs can interact with the plasma membrane in a sugar-like manner, associating with it to prevent excessive expansion and the formation of transmembrane pores [4, 24, 25]. Thus, the use of polysaccharides in combination with DMSO in cryoprotective media may improve the cryopreservation outcomes of cell suspensions composed of various cell types.

Any model is an approximation of what may occur during real cryopreservation, and this approach to measuring certain biophysical parameters of the membrane is no exception. However, it provides insight into the potential responses of different biological objects (*e. g.*, various cells within a cell suspension). Increasing the complexity of the model does not always yield satisfactory results, but addressing certain issues related to the presented modeling may bring us closer to developing effective methods for preserving complex and heterogeneous biological materials (such as bone marrow cell suspensions, umbilical cord blood cells, *etc.*).

CONCLUSIONS

In conclusion, the cryopreservation of heterogeneous cell suspensions is a complex biotechnological task that requires careful selection of cryopreservation parameters. The success of cryopreservation for such biological materials depends on membrane permeability to permeating CPAs and water, which, in turn, can predispose cells to osmotic stress, CPA toxicity, intracellular ice formation, and/or cell damage caused by the "solution effect" during extracellular ice formation. Herein, we have shown that cell osmotic changes can be assessed and Kedem-Katchalsky modeling can be used to estimate the time required for sufficient saturation of cells with DMSO in heterogenous suspensions. Averaged membrane permeability pa-

rameters (L_p, P_s, E_{a0}, and E_{a1}) were determined. This allowed us to explain the benefits of minimizing the time of exposure of cells to DMSO-containing media. Essentially, any cell type requires some time for the DMSO concentration in these cells to reach values comparable to those of the solution surrounding the cells. In this case, DMSO can exert its protective effect against the main factors causing cryodamage. However, modeling showed that for the heterogeneous suspension obtained from testicular cells, a few minutes were sufficient for saturation. This time is approximately comparable to that during which the cells remain in the solution from the moment DMSO supplement to the cryodamage onset, caused by temperature decrease. Therefore, additional exposure of these cells to DMSO before cooling is not only unnecessary but can actually lead to negative consequences, such as the effects of DMSO on membranes and various toxic effects. Such a modeling can be used in other research that deals with cells of multiple types. The membrane permeability parameters may also be used to determine optimal cooling rates, and this work is currently underway.

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МОДЕЛЮВАННЯ ОСМОТИЧНОЇ ПОВЕДІНКИ КЛІТИН
ДЛЯ ВДОСКОНАЛЕННЯ ПРОТОКОЛУ КРІОКОНСЕРВУВАННЯ

Кріоконсервування клітинних суспензій, таких як клітини інтерстицію сім'яника (КІ), які включають клітини, що відрізняються за розміром, об'ємом і складом мембран, є надзвичайно складним завданням. Кріобіологія вимагає визначення кількох параметрів для оптимізації процедури кріоконсервування, таких як коефіцієнт проникності мембрани для води (L_p) і його енергія активації (E_{a0}), коефіцієнт проникності плазматичної мембрани клітин для диметилсульфоксиду (ДМСО) (P_s) і його енергія активації (E_{a1}), а також осмотично

неактивний об'єм клітини. У роботі визначали усереднені значення L_p, P_s, E_{a0} та E_{a1} для КІ, оцінювали ступінь насичення клітин ДМСО і обґрунтували оптимальний час експозиції КІ у середовищах для кріоконсервування перед охолодженням. Кінетика зміни об'єму КІ у кріопротекторних середовищах із різною концентрацією ДМСО (0,7—2,8 М), які додатково могли містити 100 мг/мл декстрану 40 (0,7ДМСО + Д40) або 10 % ФБС (1,4ДМСО + ФБС), аналізувалась з використанням модифікованої моделі Кедема–Качальського для визначення транспортних властивостей мембран. Розраховані параметри чітко показали, що насичення клітин ДМСО відбувалося протягом кількох хвилин після його додавання і могло досягати рівноваги до початку кристалізації води у зразках. Таким чином, додаткова експозиція клітин у середовищі з ДМСО була зайвою та могла потенційно знижувати метаболічну активність КІ. Відповідно, процедуру кріоконсервування КІ можна скоротити шляхом виключення етапу експозиції без зниження життєздатності КІ у середовищах 0,7ДМСО + + Д40 та 1,4ДМСО + ФБС.

***Ключові слова*:** безсироваткові середовища, середовища без ксеногенних компонентів, декстран, коефіцієнт проникності, енергія активації, диметилсульфоксид.