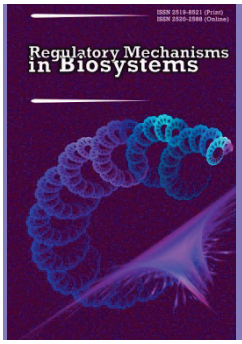

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Contribution of extracellular solute transfer to testicular cell damage

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Cryopreservation of cells involves many mechanisms of cell damage. The main general mechanisms are immediate cell damage by growing intracellular ice crystals and the adverse influence of hyperconcentrated solutions on cellular components. The formation of the solutions is triggered by intra- and extracellular ice growth. Components of the hyperconcentrated solution may enter living cells during cooling and lead to the post-hypertonic lysis of the cells on warming of the cells and on removal of cryoprotective agents. The mechanisms of entry of extracellular solutes such as Na^+ and Cl^- ions is unclear. Some studies believe that they may involve the opening of mechanosensitive ion channels when cellular membranes are stretched or squeezed. The present work has assessed the involvement of the mechanisms of ion transport across the membrane of rat testis interstitial cells in their post-hypertonic lysis and its contribution into the interstitial cell loss after incubation and cryopreservation. It has been shown that incubation in anisosmotic conditions with and without dimethyl sulfoxide lowered the indicators of interstitial cell survival. Furthermore, the gradual removal of hyperconcentrated media increased the survival of interstitial cells, which points to the osmotic mechanisms of interstitial cell damage. The mechanisms would be caused by the initial entry of inorganic ions or cryoprotective agent dimethyl sulfoxide (DMSO) into the cells. However, the use of the inhibitor of ion transport across the membrane chlorpromazine did not improve the survival of interstitial cells after incubation. DMSO, able to suppress ion transfer across the membrane, lowered the survival of cells. Interestingly, this lowering effect was less pronounced at moderately hypertonic medium (600 mOsm). Chlorpromazine did not improve the outcome of cryopreservation with 0.7 M DMSO. Thus, the contribution of the entry of inorganic solutes into the post-hypertonic IC damage was minimal after incubation, although, gradual removal of DMSO or transfer of the interstitial cells into isosmotic condition is justified. Chlorpromazine did not provide any advantages for cryopreservation of interstitial cells.

Keywords: post-hypertonic lysis; regulatory volume increase; dimethyl sulfoxide; chlorpromazine; testis; cryopreservation.

Introduction

Regulatory volume increase/decrease (RVI/RVD) are physiological cellular mechanisms by which cells can adapt to changes in osmolarity of the surrounding (Traversari & Cincotti, 2021; Olver & Benson, 2023). This mechanism involves opening of ion channels sensitive to plasma membrane distortions (stretching or squeezing) (Michelucci & Catacuzzeno, 2024). The RVI can be outlined in the following way: there is a cell volume reduction if cells are placed into hypertonic media, caused by massive water efflux from the cells. An excessive volume loss may lead to cytoskeleton alteration, changes in the interaction between the cytoskeleton and the membrane, membrane damage and cell loss. To counteract the excessive volume loss, mechanosensitive Na^+ and Cl^- membrane ion channels open and allow the entry of these osmotically active species into the cell. Thus, the volume of cell can be partially preserved. Hypotonicity results in the reverse process known as RVD. RVI (and RVD) can modify the osmotic tolerance of cells. However, in vitro, the physiological RVI may cause what is called the post-hypertonic lysis, if the cells incubated in a hyperosmotic medium are transferred fast to isosmotic one (Klbik, 2024). The post-hypertonic lysis was initially described for erythrocytes (Lovelock, 1953) but its possible involvement in cryodamage was described for other types of cells (Peckys & Mazur, 2012; Traversari & Cincotti, 2021; Olver & Benson, 2023).

Cryopreservation of cells and tissue involves the addition and removal of osmotically active CPAs, the equilibration of the CPA with the cell interior, cooling/warming. Cooling can lead to crystallization of water molecules, ice crystal growth and a progressive increase in the concentration of solutes in the cell surrounding. The entry of normally osmotically non-permeable solutes such as Na^+ and Cl^- ions through the membrane damage or by RVI into the cell is possible. Our previous work has shown that testicular interstitial cells (ICs) may be lost, even if the temperature of cryoprotective medium decreased only to –30 ºC (Pakhomov et al., 2022; Pakhomov et al., 2024). Warming and/or CPA removal from the cells can be accompanied by water entry into ICs, their swelling and loss by the mechanism generally the same as the post-hypertonic lysis. In line with these effects, inhibitors of ion transport such as ChP may potentially increase the amount of the surviving cells by lowering the entry of the inorganic ions into the cells in hyperosmolar media (Ogata et el., 1989; Semionova et al., 2017; An et al., 2023). The study of the mechanisms underlying cryodamage of ICs during cryopreservation can shed light on the possible ways of increasing osmotic tolerance of the cells and improving cryopreservation protocols.

The use of cryopreserved testicular cells and tissues including ICs may help to preserve endangered species and lines (Schlatt et al., 2003; Hutka et al., 2017; Morrell & Mayer, 2017), establish germplasm banks or compensate for androgen deficiency in fertility preservation and reproductive technologies (Holoch & Wald, 2011; Kirpatovskii et al., 2018). Many cryopreservation protocols for testicular cells and tissue include DMSO (Keros et al., 2005; Keros et al., 2007; Chen et al., 2007). Therefore, investigation of osmotic tolerance changes of ICs in the presence of DMSO is of particular interest as this may clarify the aspects connected with DMSO toxicity, osmotic activity and its cryoprotective properties. Moreover, some studies display DMSO ability to suppress the transfer of ions across the membrane (Farrant, 1972; Mironescu, 1978; Nardid et al., 2013).

The objective of the study is to evaluate osmotic tolerance of ICs in the presence of DMSO, to assess the involvement of the mechanisms of

RVI in cryodamage of these cells and the use of inhibition of membrane ion transport for the improvement of the outcome of cryopreservation.

Materials and methods

Experimental animals. Male Wistar rats form the animal house of the Institute for Problems of Cryobiology and Cryomedicine of the NAS of Ukraine (Kharkiv) were used. The animals were given the standard laboratory chow and water *ad libitum*. The experimental protocol followed the Guide for Care and Use of Laboratory Animals and was approved by the Committee for Ethics in Animal Experimentation of the Institute for Problems of Cryobiology and Cryomedicine of the NAS of Ukraine.

Isolation of testicular interstitial cells. Isolation of testis was done as described (Pakhomov et al., 2024). Cervical dislocation was used for scarification of rats. The animals' body were immersed in 75% ethanol for 5 min. The testes were isolated, decapsulated and the blood vessels were trimmed from the testes. After that, they were placed in 15-mL centrifuge tubes containing 4 mL 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 min. The tubes were placed in a thermostated shaking water bath at 90 cycles/min and 34 °C. 10 mL of collagenase-free DMEM F12 (Biowest, France) were added to each tube. After that, the seminiferous tubule mass was removed by filtration through doubled, 100 μm nylon mesh. The filtrates were centrifuged at 325 g for 3 min at room temperature. Supernatants were discarded whereas the residues were resuspended in 10 mL phosphate buffer saline (PBS). The procedure of sedimentation was repeated. Cell concentration was adjusted to 4×10^7 cells per mL with PBS. Then the cell suspensions were diluted with concentrated cryoprotective media (1:1) to reach the final concentration of additives (see below). Thus, the final concentration of cells in a sample came to 2×10^7 cells/mL.

Testing osmotic tolerance. For osmotic tolerance testing standard phosphate buffer saline (PBS) with 10-times elevated concen-tration of solutes (x10PBS) and average osmolarity of 3 Osm, and distilled water were used to prepare the solutions that on 1:1 mixing with ICs suspension gave final basic PBS osmolarities of 150, 225, 300, 600, 900, 1200 mOsm. ICs were incubated 40 minutes at 4 °C in 1 mL. The solutions may additionally contain 0.7 M DMSO (Galychfarm, Ukraine) and/or 15 μM ChP (Sigma, USA). After that, the media were removed and replaced by PBS (300 mOsm). Two types of removal of such media were used: fast and gradual. Fast removal was executed by single time addition of 5 mL of PBS. For the gradual removal, first five 200 μL-aliquots of PBS with the interval of 30 seconds were added. Then two 500 μL- and three 1 mL-aliquots of PBS were added to the samples with the same interval. Then the cells were centrifuged at 325 g for 3 min at room temperature. The supernatant was discarded. 5 mL of PBS (300 mOsm) was added. The procedure of sedimentation and supernatant removal was repeated again. Finally, the indicators of cell survival and the volume of ICs were measured.

Measurements of indicators of cell survival. For the measurements of indicators of cell survival, ICs mixed with 0.4% Trypan Blue dye (1:1) were counted in a hemacytometer chamber. The general cell survival was determined as the ratio of the number of all cells in a sample after incubation to the number of cells in PBS (300 mOsm) before incubation by 100%. Cells unstained by Trypan Blue dye were considered as cells having intact membrane. The survival of cells with the intact membrane was the ratio of the number of all 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 IC metabolic activity 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 after incubation at 4 °C and medium removal. The cells were incubated with MTT for 2 h at 35 °C. Then, ICs were sedimented at 325 g for 3 min. The supernatant was discarded. 450 μL of dimethyl sulfoxide was added to the stained pellet. Then, the samples were stirred. After 5 minutes the samples were centrifuged and the optical density (extinction) of the supernatant of the samples was measured at 530 nm. The samples containing no cells were used as a reference. The metabolic activity preservation was 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%.

Measurements of cell volume changes. Cell volume data were obtained using an AxioObserver Z1 microscope (Carl Zeiss, Germany). The microscope was equipped with a camera for acquiring images of cells in the incubation media or immediately after the removal of incubation media. Images were analyzed using an image processing program (AxioVision Rel.4.8, Germany). Since all cells remained generally spherical during the observations, the average cell diameter was calculated by measuring and averaging several of its diameters (at least 3). After that, the change in cell volume depending on time was presented as a normalized volume – the ratio of the cell volume during or after incubation to the initial cell volume at PBS (300 mOsm).

Cryopreservation. Cryopreservation was done as described (Pakhomov et al., 2022). Briefly, 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) to get the final DMSO and ChP concentration of 0.7 M and 15 μM respectively. There were also samples for comparisons that were not supplemented with ChP. The cooling was done with a programmable freezer at a rate of 1 °C/min from 4 to –80 °C. As the final temperature of the samples was reached, they were submerged into liquid nitrogen (–196 °C) and stored for a week. The samples were warmed in a water bath at 37 °C and the procedure of gradual removal of CPAs described above was carried out. After that, the indicators of IC survival were measured.

Statistical analysis. Data were represented as a median, 25th percentile, 75th percentile, maximum and minimum. The data were ranked before the Tukey test was used to reveal the differences between several groups. Similarly, the Dunnett test was used for multiple comparision with one group. Mann-Whitney U test was used for paired comparisons. $P < 0.05$ was considered significant.

Results

ICs were very sensitive to hypo-osmotic conditions as far as the deviation from isosmotic conditions (300 mOsm) by 25% (to 225 mOsm) resulted in the reduction of general cell survival and metabolic activity of the samples by 50% (Fig. 1). These indicators decreased almost linearly depending on osmolarity of concentrated PBS in a row of 300, 600, 900, 1200 mOsm. Interestingly, if ICs incubated in the presence of 0.7 M DMSO, the maximum indicators were observed in 600 mOsm-hyperosmolar solutions. Regardless of the fact, the indicators were reduced when compared with those of the samples incubated without DMSO.

The way of transferring did not play a significant role on cell survival, except in case of 600 mOsm PBS where the number of ICs were slightly higher in the sample which was gradually transferred from hypertonic medium to isotonic (Fig. 2). This finding implies possible damage of cells by osmotic swelling caused by inorganic ion entry in ICs after incubation in the medium having elevated salt concentration.

ICs are a cell suspension that includes many types of cells. One type of cells was microscopically distinct from any other cells in the suspension and had larger volume (Fig. 3). The diameter of these cells came to about 18.3–19.8 μm. They were conditionally designated as "large cells" to distinguish them from the rest of ICs, "small cells". The volume of ICs, survived incubation, depended on osmolarity of PBS in which they were incubated before transferring into isosmotic conditions (300 mOsm PBS): 900 and 1200 mOsm PBS decreased the volume of large cells significantly (Fig. 4). The way of transferring into isosmotic conditions played a role in case of 1200 mOsm with ICs being larger after fast transferring. Their volume was reduced compared with ICs incubated at isosmotic conditions. As for small cells, the characteristic dependencies, described for large cells, were not observed, probably due to high heterogeneity of the population.

As far as the volume of cells incubated at 600 mOsm remained unchanged and the decrease in IC survival was not as critically low as in the case of 900 and 1200 mOsm PBS, the next stage of the work was carried out using 600 mOsm PBS as a basic medium. We hypothesized that the indicators of IC survival in the medium with moderate hyperosmolarity (600 mOsm) would be increased at the expense of inhibition of putative

inorganic ion entry in ICs during incubation. This inhibition would potentially prevent the post-hypertonic lysis of ICs after transferring into isosmotic conditions. Theoretically, if ChP (and/or DMSO) inhibits ion transport, it should increase the survival of ICs after incubation in hypertonic conditions and explain why maximum IC survival was detected in hypertonic 600 mOsm-PBS after incubation with 0.7 M DMSO.

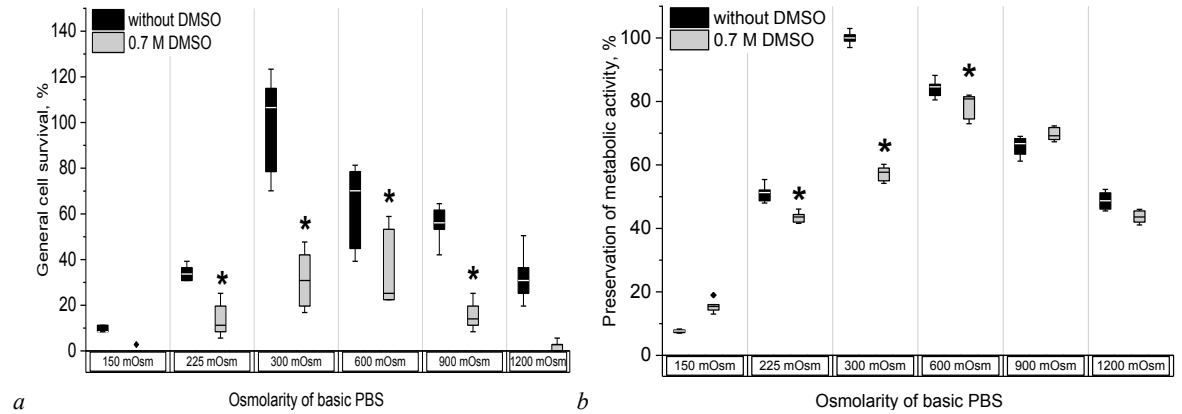

Fig. 1. Indicators of cell survival after incubation in anisosmotic conditions with and without DMSO: (*a*) general cell survival and (*b*) preservation of metabolic activity; line in the middle of the box – median, upper and lower box borders – 25% and 75% quartiles, vertical line – minimum and maximum values, circles – outliers; * – the indicators of cell survival were statistically different from the samples incubated without dimethyl sulfoxide in the solutions having the same inorganic salt osmolarity ($P < 0.05$)

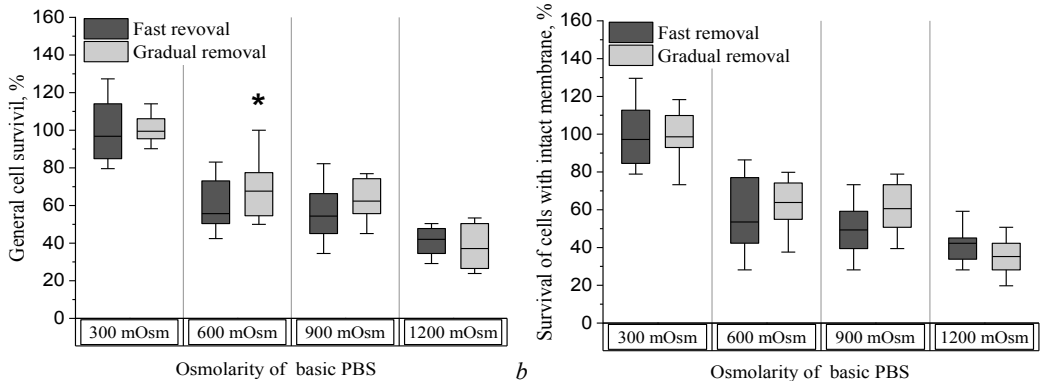

Fig. 2. Indicators of cell survival after incubation in anisosmotic conditions with and without dimethyl sulfoxide: (*a*) general cell survival and (*b*) survival of cells with intact membrane; see Figure 1; * – the indicators of cell survival were statistically different from fast removal ($P < 0.05$)

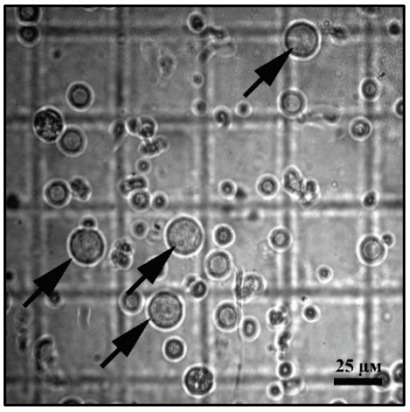

Fig. 3. The heterogeneity of IC suspension: black arrows demonstrate the large cells

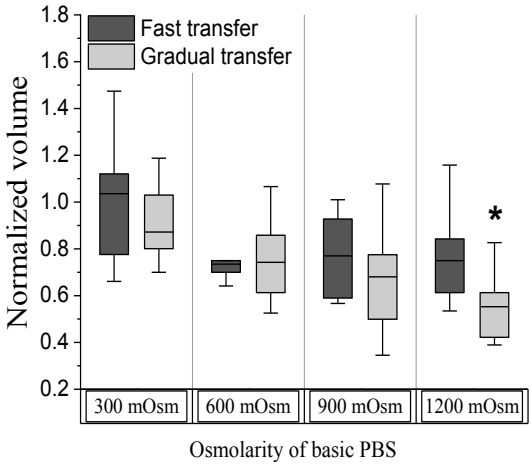

Fig. 4. The normalized volume of large ICs after incubation in hypertonic media followed by transferring in isotonic conditions; see Figure 1; * – the indicator was statistically different from fast removal ($P < 0.05$)

Our findings have shown that ICs were sensitive to the agent. For example, the least concentration of ChP that did not lower indicators of cell survival after 40 min incubation at 4 ºC was 15 μM (data not shown). Hence, it was further used for investigation of ion transport across IC membranes (Fig. 5). ChP and DMSO, as well as their combination decreased the indicators of IC survival after incubation in 600 mOsm PBS, which means they did not inhibit ion transport and/or the transport did not occur at all (or were not responsible for the cell loss). Furthermore, gradual transfer of the cells in isosmotic medium resulted in even lower survival. Gradual removal increased the survival of cells incubated with dimethyl sulfoxide.

Although ICs in PBS (600 mOsm) with ChP were somewhat smaller in size (Fig. 6a) and fast removal of ChP resulted in slightly larger volume (Fig. 6b), there were no statistically significant differences between ICs incubated in ChP and/or DMSO, as well as after the removal of the additives and transferring of the cells into isosmotic media. The use of ChP for cryopreservation of ICs with 0.7 M DMSO did not improve the outcome compared to the samples devoid of ChP (data were not shown).

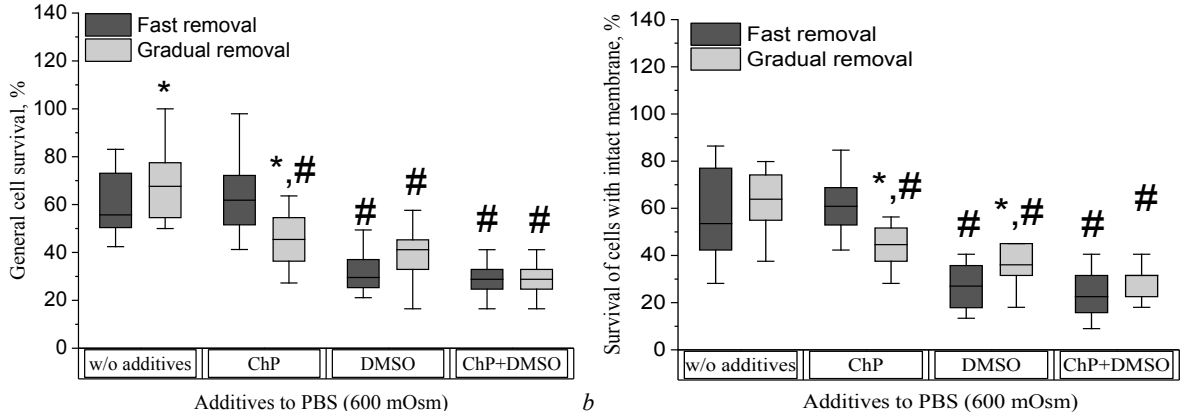

Fig. 5. Indicators of cell survival after incubation in 600 mOsm PBS with or without 15 μM ChP, or 0.7 M DMSO or any of the additives (w/o additives): (*a*) general cell survival and (*b*) survival of cells with intact membrane; see Figure 1; * – the indicators were statistically different form fast removal; # – the indicators were statistically different from PBS without additives for the same type of removal ($P < 0.05$)

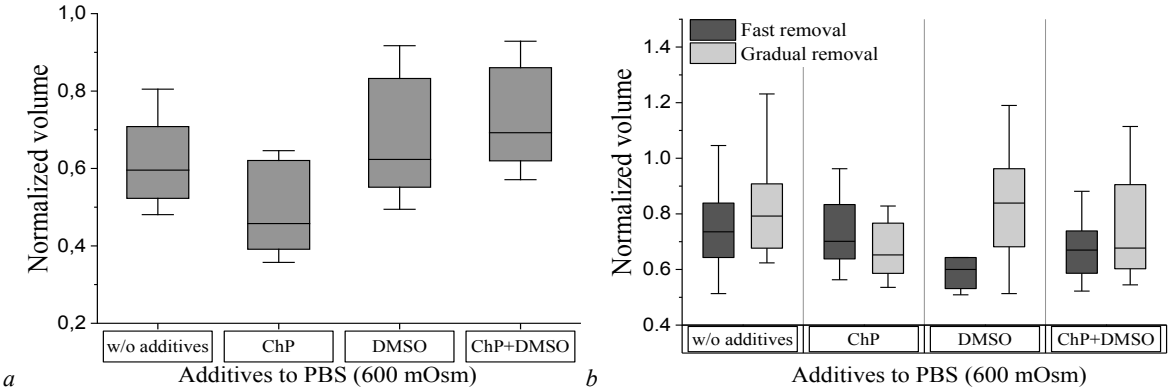

Fig. 6. Interstitial cell volume distributions: (*a*) in 600 mOsm PBS supplemented with ChP or DMSO and (*b*) after removal of additives and transferring cells into isosmotic PBS; see Figure 1

Discussion

According to Mazur's two factor theory of cryodamage, the main factors affecting cell survival are the intracellular ice and hyperconcentrated solution that form when the growth of extracellular ice progresses (Mazur et al., 1972). The effect of hypercon-centrated solutions on cells during cryopreservation is known as "the solution effect". CPAs can, to some extent, attenuate the factors. For example, DMSO can decrease the amount of ice formed at any given temperature, lower the concentration of solutes surrounding cells after initiation of extracellular phase formation, promote the formation of the amorphous phase and suppress eutectic crystallization (Meryman et al., 1971; Kratochvílová et al., 2017; Pakhomov et al., 2024). However, DMSO itself can be osmotically active and toxic. Its toxicity shows itself as thinning of the lipid bilayer, transmembrane pore formation and decrease in metabolic activity of cells (Gurtovenko & Anwar, 2007; Awan et al., 2020; Pakhomov et al., 2022). As DMSO is osmotically active and able to penetrate plasma membranes, it can cause cell volume excursions and, thus, cell damage, when added or removed from the medium with cells (Casula et al., 2019; Traversari & Cincotti, 2021).

ICs were very sensitive to hypertonic and, especially to hypotonic PBS. It is generally known that the cellular membrane is more sensitive to stretching in hypotonic solutions than to shrinkage in hypertonic ones (Gordienko & Pushkar, 1994). This explains why the indicators of IC survival in the media having twice lower osmolarity (150 mOsm) than isosmotic PBS (300 mOsm) considerably went down. Conversely, a double increase in osmolarity (to 600 mOsm) led to moderate reduction of cell survival. The presence of DMSO in incubation media decreased the indicators of cell survival when compared with basic PBSs having the same osmolarity. However, the maximal cell survival was observed not only in 300 mOsm-PBS but also in 600 mOsm, which hints at possible DMSO effects on water and ion transfer across the plasma membrane.

There is a mechanism by which cells can accommodate osmotic changes of the surrounding media known as RVI/RVI (Peckys & Mazur, 2012; Traversari & Cincotti, 2021; Olver & Benson, 2023). It states, for example, that if a cell gets into hyperosmotic conditions, the osmotic exit of water from the cell leads to a decrease in its cell volume and to membrane shrinkage. This, in turn, triggers opening mechanosensitive channels for generally impermeable Na^+ and Cl^- ions. The ions move into the cell preventing excessive loss of water and, correspondingly, cell volume reduction. As far as cryobiology is concerned, the above-mentioned solution effect may lead to the entry of normally impermeable ions into the cells, which on rapid ice melting or fast CPA removal can cause excessive cell swelling and membrane rapture, also known as the post-hypertonic lysis (Traversari & Cincotti, 2021; Olver & Benson, 2023).

Gradual transfer of ICs into isosmotic conditions was slightly beneficial for ICs incubated in 600 mOsm. PBSs with higher osmolarity apparently were too damaging to ICs. The ICs that survived incubation were not swollen. Moreover, we even observed moderately reduced cell volume of the surviving ICs after incubation, which was dependent on osmolarity of incubation media, especially in case of 900 and 1200 mOsm PBSs. This can be due to the loss of plasma membrane surface in hypertonic solutions and/or the loss of intracellular content of the surviving cells under transfer into isosmotic PBS (300 mOsm) (Kuleshova, 2006; Casula et al., 2019). Additionally, gradual removal promoted the survival of ICs incubated in 600 mOsm PBS supplemented with DMSO compared with fast removal. This also emphasizes the influence of cell volume osmotic excursions on the IC survival but, in this case, chiefly caused by the permeating CPA.

If the ICs, which did not survive incubation, had been damaged by the mechanism of post-hypertonic lysis, the inhibitors of ion transport (ChP or DMSO) would have increased the fraction of the survived cells and been potentially beneficial for cryopreser-vation by preventing the entry of normally non-permeating inorganic ions (Na^+, Cl^-) and suppressing the solution effect. The concentrations of ChP commonly used for inhibition of ion transport range from a few micromole to 600 μM (Ogata et al., 1990; Lee et al., 2017; Semionova et al., 2017). ICs were sensitive to ChP. The minimal ChP concentration that did not cause cell damage was

15 μM. However, neither 15 μM ChP, nor 0.7 M DMSO, let alone their combination resulted in any additional increase of indicators of IC survival. Moreover, if ChP had inhibited ion transport of ICs, it would have additionally reduced the volume of cells in hypertonic solutions and increased their volume on transfer into isosmotic conditions compared to the solution devoid of ChP. Although, we observed some reduction of cell volume of ICs on usage of ChP, it was not statistically significant and there were no differences in cell volume after the removal of ChP. Even if we admit that in some ICs the entry of Na^+ and Cl^- did occur, the mechanism of post-hypertonic lysis has little effect (if any) on IC survival after incubation. Whether the mechanism plays a role on cooling/warming of ICs has yet to be clarified in more in-depth research, however, considering potential toxicity of ChP and its inability to increase the survival of ICs after incubation in hypertonic media (and after cryopreservation with 0.7 M DMSO), its use for cryopreservation of testicular cells is unlikely to be beneficial.

Conclusion

In the present work, the osmotic tolerance profile was described for ICs. DMSO decreased the osmotic tolerance of ICs during incubation. The entry of inorganic ions into ICs and the loss of cells by mechanism of post-hypertonic lysis is shown to be possible. However, the contribution of the post-hypertonic lysis into the IC loss was assessed as minimal, as ChP and/or DMSO did not improve the outcome of incubation and cryopreservation of ICs. Gradual transferring of ICs into isosmotic conditions is recommended as it can reduce potential adverse osmotic effects of DMSO and other solutes on the cells during CPA addition/removal. The results of the research contribute to the understanding of mechanisms of cryodamage and cryoprotection. They open new perspectives for improving cryopreservation media and protocols for testicular cells and tissue, establishing low-temperature banks of biological material that, in turn, can be used for preservation of biological diversity and in reproductive technologies. Further studies are required to establish the contribution of other factors to cell membrane cryodamage caused by cell volume manipulations such as toxic effects of DMSO, disintegration of cytoskeleton, denaturation of membrane molecules. They are subjects for further research.

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The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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