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The development of the cell cryopreservation protocol with controlled rate thawing

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Abstract Thawing in the water bath is often considered as a standard procedure. The thermal history of samples thawed in this way is poorly controlled, but cryopreservation and banking of cell-based products require standardization, automation and safety of all the technological stages including thawing. The programmable freezers allow implementation of the controlled cooling as well as the controlled thawing. As the cell damage occurs during the phase transformation that takes place in the cryoprotectant medium in the process of freezing–thawing, the choice of warming rates within the temperature intervals of transformations is very important. The goal of the study was to investigate the influence of warming rates within the intervals of the phase transformations in the DMSO-based cryoprotectant medium on the cell recovery and to develop a cryopreservation protocol with controlled cooling and warming rates. The temperature intervals of phase transformations such as melting of the eutectic mixture of the cryoprotectant solution (MEMCS), melting of the eutectic salt solution (MESS), melting of the main ice mass (MMIM), recrystallization before MEMCS, recrystallization before MESS and recrystallization before MMIM were determined by thermo-mechanical analysis. The biological experiments were performed on the rat testicular interstitial cells (TIC). The highest levels of the cell recovery and metabolic activity after cryopreservation were obtained using the protocol with the high (20 °C/min) warming rate in the temperature intervals of crystallization of the eutectics as well as recrystallizations and the low (1 °C/min) warming rate in the temperature intervals of melting of the eutectics as well as MMIM. The total cell recovery was 65.3 ± 2.1 %, the recovery of the 3-beta-HSD-positive (Leydig) cells was 82.9 ± 1.8 %, the MTT staining was 32.5 ± 0.9 % versus 42.1 ± 1.7 %; 57.4 ± 2.1 % and 24.0 ± 1.1 % respectively, when compared to the thawing in the water bath.

Keywords Cryopreservation · DMSO · Controlled thawing · Temperature interval of phase transformation · Cell recovery

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Introduction

The production of living cells for clinical therapy often involves cryopreservation and banking. One of the basic principles of GMP is the control of all aspects of the production process. Much attention in the cryopreservation practice is paid to the regulation of cooling regimens, the composition of the medium, the procedures of the cryoprotectant adding and washing, the

temperature of the storage and transportation, while the rapid thawing is considered as standard (Hunt 2011; Li and Ma 2012; Lopez et al. 2012; Polchow et al. 2012).

Cell cryopreservation is concerned with the search for the optimal ratio between cooling and warming rates (Mazur 1966; Rall et al. 1984; Karlsson 2001; Yu et al. 2002). The warming rate at thawing plays a crucial role in the cell recovery (May and Wainwright 1985; Tao et al. 1995; Gao and Critser 2000; Parmegiani et al. 2014), especially when the intracellular ice formation took place during cooling (Leibo et al. 1978; Mazur 1984; Karlsson et al. 1993; Mazur et al. 2007; Jin et al. 2008). When the cryoprotectant is present, the dependence is stronger, because the additional phase transformations appear during freezing and thawing (Leibo et al. 1970; Souzu and Mazur 1978; Hopkins et al. 2012).

The intracellular ice formation is a latent lethal factor which is realized due to the crystal growth in the process of the long-term storage at the insufficiently low temperature (temporary recrystallization) or at the moment of cell thawing (migratory recrystallization) (Mazur 1967; Bank 1973; Vysekantsev et al. 2005; Seki and Mazur 2008).

In fact, many previous studies have shown that the rapid thawing is more preferred for cell survival (Karlsson 2001; Routledge and Armitage 2003; Jin et al. 2008). Ice is known to be more easily formed during warming than during cooling (Dowell and Rinfert 1960). If the cooling rate is too fast, the ice crystals are unable to achieve a relatively large size before the solution achieves equilibrium. Ice crystals have a large surface area per unit volume and tend to crystallize and recrystallize (depending on the degree of the solution misbalance) with thawing. Thus, the rapid thawing prevents the growth of the crystals until the moment they start to melt.

During the rapid thawing, a frozen sample is usually placed in a water bath or in air at a fixed temperature in the range from “the room temperature” up to 37–42 °C (Barth and Bowman 1988; Perseghin et al. 2000; Röllig et al. 2002; El-Naggar et al. 2006; Lopez et al. 2012; Polchow et al. 2012; Triana et al. 2013).

However, the thermal history of the samples thawed in this way is poorly controlled. Such factors as geometry, the volume of the sample cells and the heat conductivity of the containers limit the absolute cooling/warming rates and prevent a regular warming rate all over the whole volume of the sample (Dumont et al. 2003; Maffei et al. 2014). The cells located in various points of the sample are thawed with the essentially different local temperature regimens. The risk of cell death due to the overheating increases for the part of the cells located close to the container wall. The incubation of samples in the water bath is associated with potential contamination. Moreover, a large-scale cryopreservation of cell-based products requires automation of all technological stages, including thawing.

A controlled-rate freezer with programmable warming rates allows implementation of controlled thawing. Up till now, there have been no studies that determined the influence of phase transformations at the stage of thawing on the preservation of living cells, and no protocols of automated thawing have ever been developed considering the obtained data.

The goal of this study was to investigate the influence of the warming rates within the intervals of phase transformations occurring in the cryoprotectant medium at the stage of thawing on the cell recovery as well as to elaborate a cryopreservation protocol with the controlled cooling and warming rates.

In the study, the temperature intervals were determined by the method of thermoplastic deformation, and the cells from the rat testicular interstitium were used. As a result, the automated cryopreservation protocol with the controlled thawing was developed, which enabled obtaining better results for the cell recovery and the metabolic activity compared to uncontrolled thawing in the water bath.

Materials and methods

Animals

Forty Wistar rats of 200–250 g body weight were used in experiments. The rats were killed by decapitation before the testes removal. All animal experiments were performed in accordance with the protocol approved by the Institutional Bioethical Committee.

Isolation of testicular interstitial cells

The TIC were obtained as described by Teerds et al. (2007). Briefly, testes were removed, decapsulated

and subjected to the enzymatic digestion with 5 mg/mL collagenase type IV (523 U/mg, Sigma, USA) in 5 mL of DMEM (PAA, Austria) supplemented by 20 mM Hepes (Carl Roth GmbH & Co, Germany) at 34 °C in a shaking water bath (150 cycles/min) during 15 min. Following the enzymatic digestion, the equal volume of DMEM/Hepes medium was added, and the tubular fragments were allowed to settle under the unit gravity for 5–10 min. The supernatant was collected, filtered through the strainer with a pore diameter of 100 μm, and centrifuged (400*g*) for 3 min.

To eliminate the spermatozoa, the erythrocytes and the cell debris obtained suspension was layered over the 30–40 % sucrose density gradient and centrifuged (400*g*) for 7 min. After centrifugation, the cells were harvested from the broadly distributed zones of the gradient between 30 and 40 % layers and washed three times with medium. The TIC were kept on ice before cryopreservation.

Cryopreservation

The TIC were cryopeserved in DMEM/Hepes medium containing 10 % DMSO (v/v). The aliquots of the cell suspension (0.5 mL) with the concentration of $5-6 \times 10^7$ cells/mL were placed into 1.8 mL cryovials (Nunc, Life Technologies, Denmark), and an equal volume of 2× concentrated cryoprotectant medium was added dropwise. The samples were cooled in the controlled-rate freezer (Cryoson, Germany) immediately after the DMSO dilution.

To control the temperature during freezing one cryovial was equipped with a copper-constantan thermocouple.

Thawing

The controlled thawing of the TIC was performed in the controlled-rate freezer (Cryoson, Germany) with programmable warming rates and the monitoring of the temperature during the warming process by a copper-constantan thermocouple, which was placed in the reference sample.

The set of the samples were thawed from −196 °C to the room temperature in a water bath at 37 °C with the uncontrolled warming rate (control).

The cryoprotectant was removed by doubling the volume until 5 mL of DMEM/Hepes medium had been added to 1 mL of the sample during step by step dilution. After this, the cells were washed twice for 3 min by centrifugation (400*g*).

The cell recovery, viability and metabolic activity assays were conducted immediately after thawing.

Determination of temperature intervals for controlled freezing–thawing

The temperature intervals for the controlled freezing–thawing were determined by the method of the thermoplastic deformation, which is a special way of the thermo-mechanical analysis. This method makes it possible to measure the temperature intervals of phase and structure transformations taking place in the frozen cryoprotectant solution when a constant external deforming tension is applied under heating (Osetsky 2009; Gurina et al. 2011).

The samples containing the cryoprotectant solution were placed into the deformation device and frozen with the constant cooling rate down to −160 °C. After the exposure of the samples at this temperature for 10 min, the constant external deforming tension was applied. The value of the deforming tension depends on the concentration of the cryoprotectant agent in the medium. After applying the force to the sample, its temperature was increased at a constant rate. The thermoplastic curve was recorded by a chart recorder Endim 622.01 (VEB MS, Germany) in the regimen of pure shear in the deformation-temperature coordinates.

Determination of cell recovery by trypan blue staining

For staining, 0.05 mL of TIC was mixed with an equal volume of the 0.4 % trypan blue solution. The cells were counted using a hemacytometer and a light microscope. The number of the non-viable (stained) and viable (trypan blue excluded) cells were counted. The cell recovery was calculated as the percentage of the number of the viable cells obtained after thawing divided by the number of the viable cells before freezing.

Determination of cell viability by fluorescent diacetate staining

Viable cells are known to have the ability to the active fluorescent diacetate (FDA) uptake which is hydrolysed in the cytoplasm by non-specific esterase to

fluorescein. FDA inclusion assay was performed according to the methods of Coco-Martin et al. (1992). The stock solution of FDA (1 mg/mL, Sigma) was prepared in the DMSO. The stock solution was diluted 100 times by the PBS. In the assay, 5 µL of FDA solution were added to 1 mL of TIC suspension containing about 10^6 cells. The cells were incubated at the room temperature for 5 min and then analyzed on a BD FACSCalibur (BD Bioscience) flow cytometer using CellQuestPro software.

Determination of steroid-producing Leydig cells by 3beta-hydroxysteroid dehydrogenase staining

The histochemical staining of the 3beta-hydroxysteroid dehydrogenase (3beta-HSD) was carried out to detect the steroidogenic enzyme in Leydig cells. The staining was carried out as described by Klinefelter et al. (1987). In brief, 50 µL of TIC suspension was placed on the slides and kept at the room temperature for about an hour. After cell drying, the slides were coated with the staining solution prepared by mixing solutions A and B. Solution A: 1 mg of nitro-blue tetrazolium (Sigma) was dissolved in 0.6 mL of dehydroepiandrosterone (Sigma, 1 mg/mL in DMSO). Solution B: 10 mg β-NAD (Sigma) was dissolved in 9.5 mL PBS. The cells were stained for 90 min, then washed in the distilled water and fixed in the PBS containing 10 % formalin and 5 % sucrose. The slides were coated with a solution of PBS/glycerol (1:1) and covered by the cover-glass. The 3-beta-HSD-positive cells were identified as cells with dark blue formazan granules. The samples were examined under the microscope MEIJU Techno (Japan). The number of the stained cells was counted using ocular 12.5 and objective 20.

Determination of cell metabolic activity by MTT assay

The conversion of methylthiazole tetrazolium to a colored formazan product reflects the overall cell metabolic activity which is associated with mitochondrial, cytoplasm and non-mitochondrial membranes including the endosome/lysosome and plasma membrane (Berridge et al. 2005).

In the assay, 50 µL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma) solution in PBS (5 mg/mL) was added to 500 µL of the TIC suspension. The cells were incubated for 4 h at 35 °C. After incubation, the MTT solution was removed by centrifugation and 450 µL of DMSO were added to the pellet. The cells kept with DMSO for 5 min followed by stirring. Finally, the absorbance of the cell lysate was measured at 490 nm. The samples containing no cells were used as a control, after all experimental procedures including sedimentation and dilution. The optical density was directly proportional to the metabolic activity of cells.

Statistical analysis

18 experiments have been carried out. Ten batches of cells were used with 2 replicates per experiment. All data are presented as a mean ± SD. The statistical significance of differences was calculated with a two-tailed unpaired Student's *t* test or a one-way ANOVA with Bonferroni's multiple comparison test. $P < 0.05$ was considered as statistically significant.

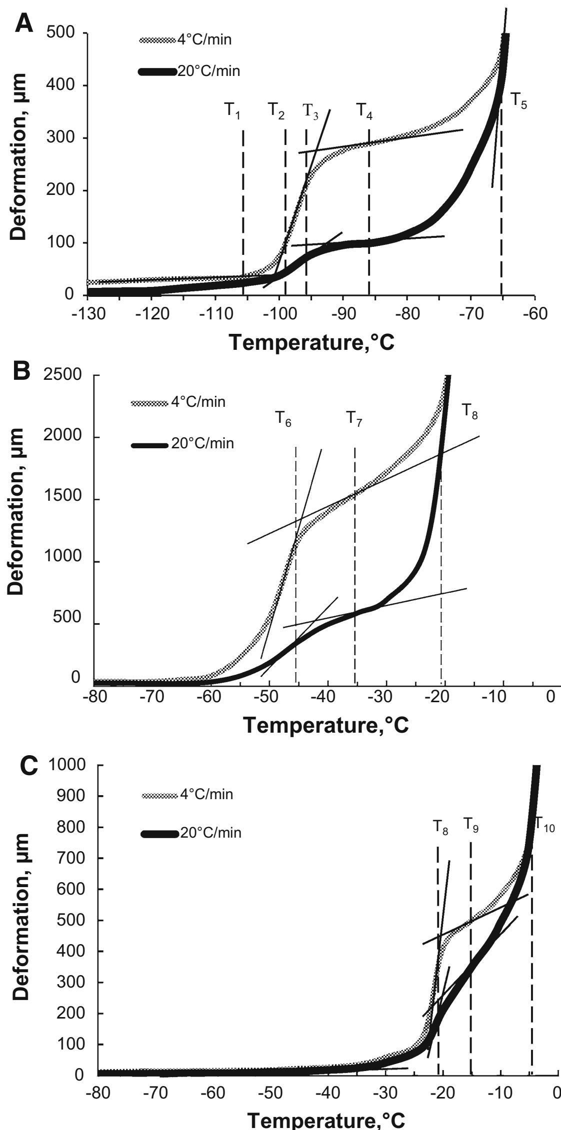
Results

Determination of temperature intervals by method of thermoplastic deformation

In our previous study (Gurina et al. 2011), a principle for determining temperature intervals of phase transformations that occur in the frozen sample during warming was demonstrated on the example of a typical thermoplastic curve for aqueous cryoprotectant (DMSO) solution. This intervals include the devitrification of the amorphous phase, melting of the eutectic mixture of the cryoprotectant solution (MEMCS), melting of the main ice mass (MMIM) as well as recrystallization before the MEMCS (rMEMCS) and recrystallization before the MMIM (rMMIM).

For the preparation of the cryoprotectant solution, the PBS or physiological solution is more often used than distilled water or various culture media. The thermoplastic curves for the PBS- or medium-based cryoprotectant solutions have characteristic features which are caused by the presence of another phase transformation associated with the melting of the eutectic of aqueous solution of NaCl.

The eutectic temperature, obtained by the method of thermoplastic deformation for saline and most



◀**Fig. 1** The thermoplastic curves for 10 % Me_2SO solution in the medium of 199 with 20 mM Hepes. The samples of the solution were frozen with various cooling rates (4, 20 °C/min). Then, the constant warming rate 1 °C/min and constant external deforming tension $\sigma = 4 \times 10^5$ kg/m^2 (**a**), 0.4×10^5 kg/m^2 (**b**), 0.13×10^5 kg/m^2 (**c**) were applied. The inflections correspond to the processes of: T_1–T_2—devitrification of the amorphous phase, T_3–T_4—rMEMCS, T_4–T_5—MEMCS, T_6–T_7—rMESS, T_7–T_8 – MESS, T_8–T_9—rMMIM, T_9–T_{10}—MMIM. The digital values of the initial and final temperature intervals of T_3–T_4 (−97 to −87 °C) and T_4–T_5 (−87 to −65 °C), T_6–T_7 (−45 to −37 °C) and T_7–T_8 (−37 to −21 °C), T_8–T_9 (−21 to −15 °C) and T_9–T_{10} (−15 to −6 °C) do not depend on the sample's cooling rate. The degree of recrystallization and, accordingly, the intensity of inflections on the curves depend on the cooling rate in the temperature intervals of T_3–T_4, T_6–T_7, T_8–T_9. The differences in the length of the part of the curves before the beginning of recrystallization (temperatures T_3, T_6, T_8) are caused by the decreasing plastic flow rate, which testifies to the differences in the sample's structures formed at the cooling stage

frequently used in cryobiology culture media, (medium 199, DMEM, Hanks) have practically the same values which coincide with the eutectic temperature for saline −21.2 °C (T.M. Gurina and Kyryliuk 2012).

To elaborate the optimal protocol for cryopreservation, the phase transformations associated with the presence of cryoprotectant agents as well as the other components of cryoprotectant solution should be considered. The required set of the thermoplastic curves for determining the temperature intervals of phase transformations in the cryoprotectant medium used for cryopreservation of the TIC is demonstrated in Fig. 1. The graphs are presented on a scale which is most suitable to determine the temperature intervals of phase transformations. Each of the figure curves (Panels A, B, C) corresponds to a value of the optimal external deforming tension σ, kg/m^2 (4×10^5; 0.4×10^5 and 0.13×10^5, respectively).

The external deforming tension is the factor by which the degree of thermoplastic curve inflections associated with phase transformations of certain components of cryoprotectant medium can be controlled. The curves in Fig. 1 were obtained at the same heating rate 1°/min but with different cooling rates: 4°/min and 20°/min.

Panel A (Fig. 1) allows to select the temperature intervals of the devitrification of the amorphous phase (T_1–T_2), the rMEMCS (T_3–T_4) and the MEMCS (T_4–T_5). For cryoprotectant medium used for the TIC cryopreservation the temperature intervals (T_3–T_4) and (T_4–T_5) have the following numerical values: −97 to −87 °C and −87 to −65 °C.

Panel B allows determining the temperature intervals of the recrystallization before the melting of the eutectic salt solution (rMESS, T_6–T_7) and melting of the eutectic salt solution (MESS, T_7–T_8). The numerical values (T_6–T_7) and (T_7–T_8) for the given cryoprotectant medium are −45 to −37 °C and −37 to −21 °C, respectively.

Panel C allows finding the temperature intervals of the rMMIM (T_8–T_9) and MMIM (T_9–T_{10}). The

temperatures of the ending of the process of recrystallization before the MMIM and the beginning of the MMIM often coincide. The numerical values of the temperature intervals of (T_8–T_9) and (T_9–T_{10}) are −21 to −15 °C and −15 to −6 °C, respectively. According to Panel C, the temperature interval of the MESS can also be determined.

Thus, the variation of the magnitude of the external deforming tension σ allows determining the temperature intervals of the eutectic melting of specific components of the solution and the melting of the bulk of ice by a thermoplastic curve as well as the temperature intervals of recrystallization before each of these types of melting. The limits of the temperature interval of recrystallization do not depend on the cooling rate, but the magnitude of deformation of the sample before the recrystallization process has greater numerical values for lower cooling rate (Fig. 1, Panels A, B, C).

Impact of the controlled warming in the temperature intervals of the rMEMCS and the MEMCS on TIC recovery

The first set of experiments involved studying the effect of the warming rate in the temperature intervals of phase transformations associated with the presence of DMSO in the cryoprotectant medium. These are the temperature intervals of the rMEMCS (T_3–T_4) and MEMCS (T_4–T_5).

The freezing–thawing of the samples was carried out according to protocols listed in Table 1. From the room temperature to 0 °C, all of the samples were cooled for 15 min, which corresponds to a cooling rate of 1–1.5 °C/min, depending on the ambient temperature. The values of cooling and warming rates in the temperature intervals from 0 °C to T_5 are the same and equal to 1 °C/min. At temperatures below T_5 the combination of the controlled slow (1 °C/min) and fast (20 °C/min) cooling and warming rates was adjusted in order to alternately determine their relevance to each of the considered temperature intervals.

The cooling rate in the temperature interval of (T_4–T_5) on the stage of freezing was 20 °C/min for protocols 1-1, 1-2, 1-3 and 1 °C/min for protocols 1-4, 1-5, 1-6. The protocols of thawing vary in the warming rate in the temperature interval of MEMCS: 20 °C/min for 1-3, 1-6 and 1 °C/min for 1-2, 1-5. For comparison, two batches of cells (1-1 and 1-4) were thawed in a water bath at 37 °C (non controlled warming rate).

The comparison of the TIC recovery after cryopreservation according to protocols 1-1 and 1-4, 1-2 and 1-5, 1-3 and 1-6 makes it possible to assess the influence of the cooling rate in the temperature interval of (T_4–T_5) on the results of cryopreservation at the same warming regimen.

The data shown in Fig. 2 allow to estimate efficiency of the protocols with controlled rates of freezing and thawing (1-2, 1-3, 1-5 and 1-6) as compared with protocols in which uncontrolled warming rate was used (1-1 and 1-4). The post-thaw TIC recovery was significantly higher (40.6 ± 1.7) % using the protocol of freezing with the high (20 °C/min) cooling rate in the temperature interval of (T_4–T_5) in combination with controlled high (20 °C/min) warming rate in the temperature interval of the

Table 1 Protocols of freezing and thawing, which were used in the first set of TIC cryopreservation

Temperature interval (°C)	Protocol					
	1-1	1-2	1-3	1-4	1-5	1-6
Cooling rate within temperature interval, °C/min						
22 to 0	1.5	1.5	1.5	1.5	1.5	1.5
0 to −65	1	1	1	1	1	1
−65 to −87	20	20	20	1	1	1
−87 to −97	1	1	1	1	1	1
−97 to −196	Plunging into liquid nitrogen					
Warming rate within temperature interval, °C/min						
−196 to −97	NC	≈10	≈10	NC	≈10	≈10
−97 to −87	NC	20	20	NC	20	20
−87 to −65	NC	1	20	NC	1	20
−65 to 20	NC	1	1	NC	1	1

NC Non controlled warming rate

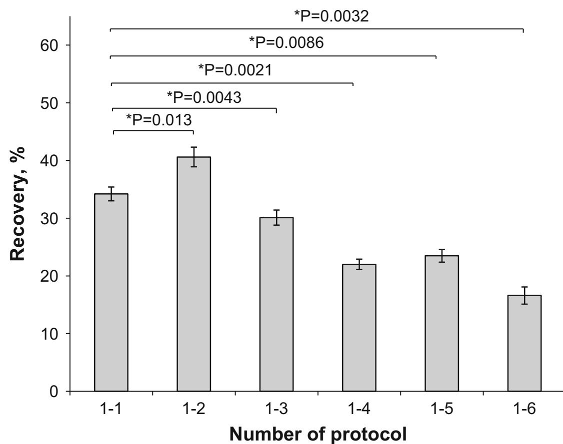


Fig. 2 Post-thaw recovery of TIC. The TIC were cryopreserved in the cryoprotectant solution (10 % Me_2SO in DMEM/Hepes) using protocols listed in the Table 1. The *bars* show the mean (±SD), n = 10 batches of cell preparation. The *asterisk* indicates a significant difference of recovery from the corresponding parameter obtained when following protocol with non controlled thawing at $P < 0.05$

rMEMCS and the low (1 °C/min) warming rate in the interval of MEMCS (protocol 1-2).

The reduction in the percentage of recovery was observed in the cells cryopreserved using protocols 1-4 (22.0 ± 0.9 %), 1-5 (23.5 ± 1.5 %) and 1-6 (16.6 ± 1.5 %), in which the low cooling rate 1 °C/min was used at the freezing stage in the entire temperature range. The results did not depend on the warming regimen.

Impact of the controlled warming in the temperature intervals of the rMESS and rMMIM as well as in the intervals of MESS and MMIM on the TIC recovery

In the second set of experiments, the effect of the warming rate in the temperature interval located above MEMCS as in the rMESS (T_6–T_7), the MESS (T_7–T_8), the rMMIM (T_8–T_9) and the MMIM (T_9–T_{10}) was examined. The experimental procedure, the principle of the selection of the numerical values of cooling, warming and its combinations in the protocols of these sets are similar to the previous one. The difference between sets of experiments is that the TIC were frozen only to −65 °C followed by thawing according to the protocols with controlled or non-controlled warming rates.

Table 2 Protocols of freezing and thawing, which were used in the second set of TIC cryopreservation

Temperature interval (°C)	Protocol					
	2-1	2-2	2-3	2-4	2-5	2-6
Cooling rate within temperature interval, °C/min						
22 to 0	1.5	1.5	1.5	1.5	1.5	1.5
0 to −65	1	1	1	1	1	1
Warming rate within temperature interval, °C/min						
−65 to −37	NC	20	1	20	20	20
−37 to −15	NC	1	1	20	1	20
−15 to 20	NC	20	1	1	1	20

NC Non controlled warming rate

According to our experimental data (Fig. 1, Panels B and C) after MEMCS (above the $T_5 = -65$ °C), no phase transformations occur in the sample during heating up to the rMESS (T_6–T_7) and the MESS (T_7–T_8), which are due to the presence of DMEM in the cryoprotectant medium. Therefore, during thawing with controlled rate the uniform temperature interval of −65 to −37 °C was examined. Considering the specifics of the recrystallization process and time constraints, a fast warming rate was used in this interval. However, for the sake of comparison, a slow warming (protocol 2-3) was also tested in this temperature interval. The temperature intervals T_7–T_8 and T_8–T_9 were merged together of −37 to −15 °C due to the small extension of the T_8–T_9.

The protocols with controlled rates of freezing–thawing in the temperature intervals above MEMCS are listed in the Table 2.

The results of our study demonstrate (Fig. 3) that the TIC recovery after cryopreservation was significantly higher using protocols 2-4 and 2-6 compared to non-controlled thawing in water bath (protocol 2-1). Taking into account the highest rate of the TIC recovery, protocol 2-4 was chosen as a basic for these temperature intervals when developing the total TIC cryopreservation protocol with controlled freezing–thawing rates.

Impact of cryopreservation with the controlled freezing–thawing rates on the TIC recovery

Considering the results of the previous sets of experiments concerning the impact of the controlled warming rates in the above-mentioned temperature

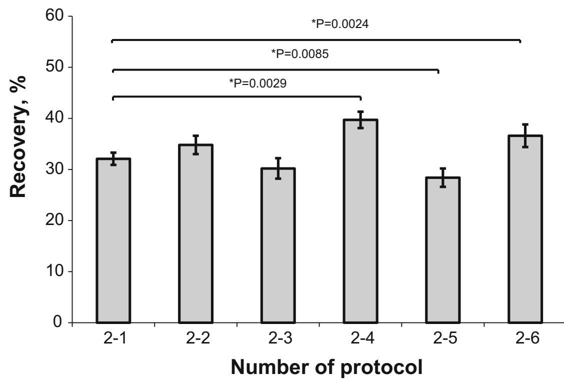


Fig. 3 Post-thaw recovery of TIC. The TIC were cryopreserved in the cryoprotectant solution (10 % Me_2SO in DMEM/Hepes) using protocols listed in the Table 2. The *bars* show the mean (±SD), n = 10 batches of cell preparation. The *asterisk* indicates a significant difference of recovery from the corresponding parameter obtained following protocol with non controlled thawing

intervals, a total of the TIC cryopreservation protocols with controlled cooling-warming rates at all subzero temperature ranges were examined. In all variants of total cryopreservation protocols (Table 3), the cooling rates were the same, but warming rates were different.

The non-controlled thawing (protocol 3-1) was used as a control. In protocol 3-4, the warming rates in each of the above temperature intervals were selected as the better of the two previous sets of experiments. The protocols of comparisons were 3-2, 3-3, 3-5 and 3-6.

A distinctive feature of the protocol 3-5 was the use of a constant high warming rate (20°/min) throughout the temperature range from −97 to 20 °C. Protocols 3-2, 3-3 and 3-6 are different from each other in the variants of high and low warming rates in the temperature intervals located above MEMCS.

The increased interest in this temperature area is due to the several types of melting that occur therein and the recrystallizations preceding them, which follow each other in a relatively narrow temperature interval. For thenculture medium, the eutectic melting and the preceding recrystallization are localized in the inter-crystalline interlayers, while the melting of the ice bulk and related recrystallization are in the entire volume of the sample.

Furthermore, these processes should be different in the degree of damage on the cells, which is associated with a very small concentration of NaCl in saline or culture medium. Meanwhile, the melting of the eutectic of aqueous NaCl solution and the preceding recrystallization occur directly in the intercrystalline interlayers, in which cells concentrate during freezing. At this stage of the study, it was important to determine the contribution of each of these processes as an unfavorable factor in the cryopreservation of cells, and to assess the possibility of reducing their damaging effects by using of controlled warming rate.

The results of the TIC recovery after cryopreservation in accordance with protocols with controlled (3-2, 3-3, 3-4 and 3-5) and non-controlled (3-1) warming rates are shown in Fig. 4.

Table 3 Total TIC cryopreservation protocols with controlled cooling and warming rates

Temperature interval (°C)	Protocol					
	3-1	3-2	3-3	3-4	3-5	3-6
Cooling rate within temperature interval, °C/min						
22 to 0	1.5	1.5	1.5	1.5	1.5	1.5
0 to −65	1	1	1	1	1	1
−65 to −87	20	20	20	20	20	20
−87 to −97	1	1	1	1	1	1
−97 to −196	Plunging into liquid nitrogen					
Warming rate within temperature interval, °C/min						
−196 to −97	NC	≈10	≈10	≈10	≈10	≈10
−97 to −87	NC	20	20	20	20	20
−87 to −65	NC	1	1	1	20	1
−65 to −37	NC	20	1	20	20	20
−37 to −15	NC	1	1	20	20	20
−15 to 20	NC	20	1	1	20	20

NC Non controlled warming rate

Figure 4 shows that protocols 3-2, 3-4 and 3-6 with controlled warming rates provide a significantly better result compared to the others. The percentage of the TIC recovery using protocols 3-2, 3-4 and 3-6 was 48.6 ± 2; 65.3 ± 2.1 and 50.1 ± 2.2 % respectively versus 42.1 ± 1.7 % in the samples thawed with a non-controlled rate (protocol 3-1).

Protocol 3-6 is simpler and easier to reproduce in the programmable freezer, but the final choice should be dependent on the type of cells to be cryopreserved, because protocols 3-4 and 3-6 differ in the distribution of the low and high warming rates in the temperature range above −37 °C associated with the MMIM and with the subsequent active cell rehydration at this stage. Certainly, it is difficult to achieve high percentages of undamaged cells without taking into account their individual properties, but even application of high constant warming rate (20°/min) in the temperature range above −60 °C (protocol 3-6) provides a large TIC recovery compared to protocol with non-controlled warming in a water bath.

Impact of cryopreservation on the 3-beta-HSD positive (Leydig cells) recovery

The Leydig cells recovery after TIC cryopreservation using total protocols was assessed using the 3-beta-HSD staining, because this enzyme takes a key role in the synthesis of testosterone. Figure 5 represents the contribution of the controlled warming rate at each temperature interval (protocols 1-2, 2-4, 2-6, 3-4, 3-6) to the total TIC as well as the HSD-positive cell recovery compared with the thawing in a water bath (protocols 1-1, 2-1, 3-1). The numbers of protocol with controlled warming rates in the Fig. 5 correspond to the best freezing–thawing regimens presented in the Table 1 (protocol 1-2), 2 (protocols 2-4, 2-6), and 3 (protocols 3-4 and 3-6).

Interestingly, the recovery of the 3-beta-HSD-positive cells following the protocols with the controlled warming rates above MEMCS (protocols 2-4, 2-6, 3-4, 3-6) was significantly higher compared to the total preservation of the cells in the suspension. A similar tendency was observed for samples cryopreserved using protocols with controlled warming rates in the temperature interval of CEMCS (protocols 1-1 and 1-2).

The highest post-thaw recovery of the 3-beta-HSD-positive cells (82.9 ± 1.8 %) was demonstrated following protocol 3-4 versus 57.4 ± 2.1 % for cells thawed in a water bath (protocol 3-1). Thus, the application of the controlled rates of warming has a definite advantage in the Leydig cell preservation compared to the thawing in a water bath.

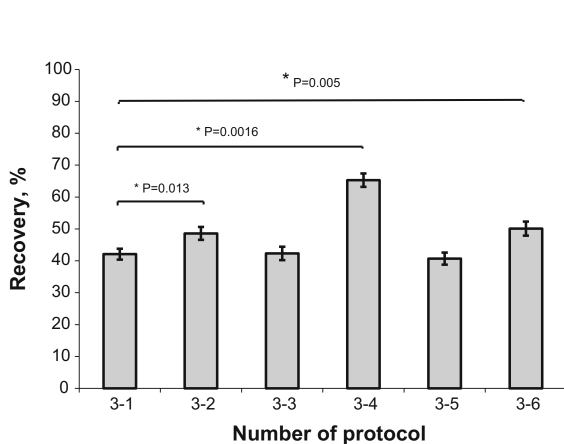


Fig. 4 Post-thaw recovery of TIC. The TIC were cryopreserved in the cryoprotectant solution (10 % Me_2SO in DMEM/Hepes) using protocols listed in the Table 3. The *bars* show the mean (±SD), n = 10 batches of cell preparation. The *asterisk* indicates a significant difference of recovery from the corresponding parameter obtained following the protocol with non controlled thawing

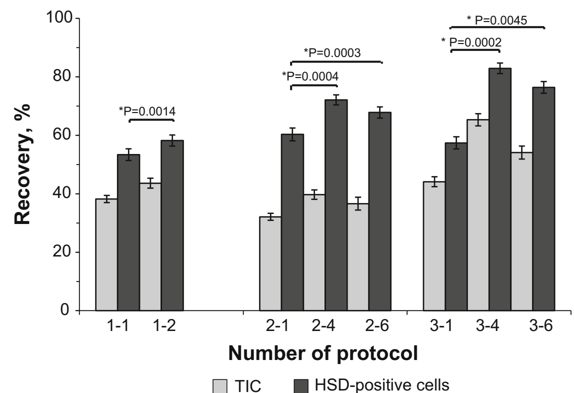


Fig. 5 Post-thaw total TIC and HSD-positive Leydig cell recovery. The TIC were cryopreserved in the cryoprotectant solution (10 % Me_2SO in DMEM/Hepes) using the protocols with the controlled (protocols 1-2, 2-4, 2-6, 3-4 and 3-6) and non controlled (protocols 1-1, 2-1, 3-1) warming rates. The *bars* show the mean (±SD), n = 10 batches of cell preparation. The *asterisk* indicates a significant difference of Leydig cell recovery from the corresponding parameters obtained following the related protocol with non controlled thawing

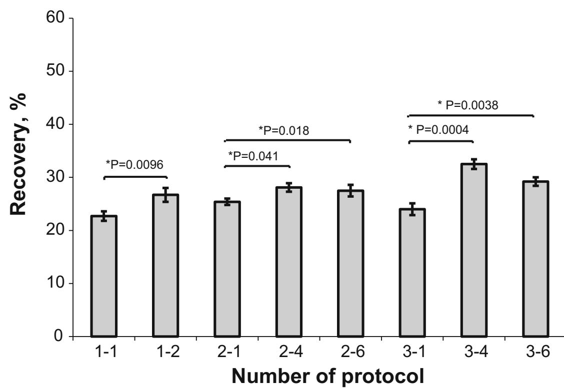


Fig. 6 MTT staining of TIC. The TIC were cryopreserved in the cryoprotectant solution (10 % Me_2SO in DMEM/Hepes) using the protocols with the controlled (protocols 1-2, 2-4, 2-6, 3-4 and 3-6) and non controlled (protocols 1-1, 2-1, 3-1) warming rates. The *bars* show the mean (±SD), n = 10 batches of cell preparation. The *asterisk* indicates a significant difference of recovery from the corresponding parameter obtained following the related protocol with non controlled thawing

Impact of cryopreservation on the metabolic activity of the TIC

Figure 6 depicts the data for the MTT staining of the TIC. The results confirmed the advantage of the controlled warming rates (protocols 1-2, 2-4, 2-6, 3-4 and 3-6) as compared with the thawing in a water bath (protocols 1-1, 2-1 and 3-1). According to the assay, the highest staining in reaction with MTT was obtained for the total protocol 3-4 (32.5 ± 0.9 %) compared to all other protocols, including the final protocol 3-1 with uncontrolled warming (24.0 ± 1.1 %).

Impact of cryopreservation on the FDA uptake

The impact of the total cryopreservation protocols on the uptake of FDA by the viable TIC is shown in Fig. 7. According to the intensity of green fluorescence, the TIC were distributed on two subpopulations: the cells with high and low fluorescence. The high fluorescence related to the active incorporation of the FDA by the cells and its hydrolyzation to fluorescein, while low fluorescent cells represent the dead cells.

Comparing the outcome of the cryopreservation protocols with the uncontrolled (protocols 3-1) and controlled (protocols 3-4 and 3-6) warming rates, the highest percentages of the TIC with the FDA staining (89.9 ± 9.2 %) were seen in the samples, which were cryopreserved according with protocol 3-4. This result

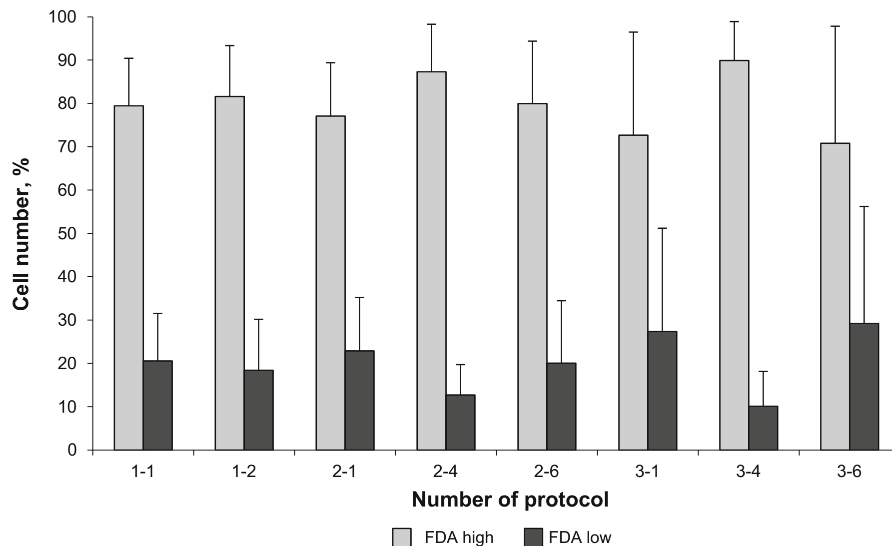


Fig. 7 FDA staining of TIC. The TIC were cryopreserved in the cryoprotectant solution (10 % Me_2SO in DMEM/Hepes) using protocols with the controlled (protocols 1-2, 2-4, 2-6, 3-4 and 3-6) and non controlled (protocols 1-1, 2-1, 3-1) warming rates. The *bars* show the mean (±SD), n = 10 batches of cell preparation

was in agreement with data obtained in the trypan blue and MTT assays (Figs. 5, 6).

Discussion

The cooling and warming regimens play the determining role for obtaining high values of post-thaw cell recovery and survival. The efficiency of warming regimen depends directly on the initial concentration of the cryoprotectant and on the regimen of the sample cooling (Harris and Griffiths 1977; Akhtar et al. 1979; Mazur 1984; Pegg et al. 1984).

There are several current approaches to thawing of the cryopreserved biospecimens. Besides the convective warming as the most commonly used technique, they include warming by microwave illumination (Guttman et al. 1980), IR laser pulses (Jin and Mazur 2015), radiofrequency excited magnetic nanoparticles (Etheridge et al. 2014). Most of them are oriented to the biological specimens frozen at ultrarapid speed, since it was previously found that the faster the warming the higher the survival for such type of frozen samples (Jin et al. 2008; Seki and Mazur 2009; Mazur and Seki 2011). However, the optimal warming rate for slowly cooled objects is less amenable to prediction (Fuller et al. 2003).

The cryoprotectant medium is known to be a mandatory component in the cryopreservation procedure. The ultimate cell recovery after the freezing–thawing procedure is dependent on the characteristics of the medium and the number of supplements therein. During the crystal ice formation, cells are placed in the narrow intercrystalline layers of unfrozen solution which mainly consist of the cryoprotectant (Mazur 1984; Maeno 1988). Therefore, at the stage of cooling, cells are influenced by the processes of crystallization of the eutectic salt solution and crystallization of the eutectic mixture of the cryoprotective solution. At the stage of warming, the processes corresponding to them go in the opposite direction: melting of the eutectic mixture of the cryoprotective solution and the MESS with the recrystallization preceding in both cases. During cooling, the main bulk of water forms a crystalline matrix until the eutectic temperatures of NaCl/water solution as well as the cryoprotectant solution. Thus, at lower temperatures it cannot influence the cells in the intercrystalline layers directly. For this reason, it is very important to control cooling-warming rates within the temperature intervals of crystallization (melting) of eutectic mixtures and other structural transformations occurring in the intercrystalline layers, in particular, in the recrystallization process at the stage of thawing.

Up till now, insufficient attention has been paid to the issues related to the cell injury at the stage of thawing. The phenomenon of melting of physiological solution or culture medium eutectic, which are often used as components of cryoprotectant solutions, are practically neglected when developing protocols of freezing and thawing.

The effect of recrystallizations, which are before the melting of NaCl/water solution eutectic and MMIM, on mechanical injury of cells is not studied yet. As the recrystallization is a process of the activation type, the rates of cooling and warming directly influence the kinetics of its proceeding. The destructive effect of recrystallization is neutralized to some extent by the viscosity of intercrystalline layers and relatively low temperatures corresponding to these phase transformations (according to our findings between −45 and −37 °C).

At first glance, the rMMIM seems unable to produce an essential mechanical impact if the cells are mainly concentrated in the intercrystalline layers. However, after MMIM the layers viscosity decreases considerably that may result in a mechanical injury of cells due to the collapse of layers by large ice crystals. Up to temperature −21 °C, certain strength is provided by eutectic of NaCl/water solution, which being in a solid phase acts as a “reinforcing mesh”. After the melting of the eutectic of the NaCl/water solution, the viscosity decreases so that it cannot prevent cell damage by the enlargement of ice crystals during recrystallization.

The present work provides a basis for rational selection of warming rates for programmable cryopreservation of slowly cooled cells. A distinctive feature of our approach is the account of peculiarities of the physical processes taking place in the temperature intervals associated with phase transformations in the cryoprotectant medium during heating (MEMCS, MESS, MMIM, rMEMCS, rMESS and rMMIM). Such temperature intervals could be determined by thermo-mechanical analysis. This should be done for each cryoprotectant medium, because boundaries of temperature intervals are depended on composition of medium (type of cryoprotectant, vehicle

solutions, additives etc.). The variation of the warming rates in these temperature intervals allows the inhibition of the mechanical damage associated with the structural change during phase transformations. The absolute numerical values of the warming rates (1 and 20 °C/min) were chosen based on the results of our previous work, which was focused on the determination of cooling rates within the temperature intervals of phase transformations (Gurina et al. 2011).

As a result, the thawing with the controlled warming rate showed the recovery of the TIC higher than with the thawing in the water bath, especially, for the population of Leydig (3-beta-HSD positive) cells (see Fig. 5). The highest levels of the TIC recovery and metabolic activity after cryopreservation were obtained using a protocol 3-4. This protocol involved the use of controlled rates on both the cooling and the warming stages, while the numerical values of the rates were varied depending upon the type of phase transformation (see Table 3).

There are several works (Rajotte and Mazur 1981; Henry et al. 1993; Koshimoto and Mazur 2002; Yu et al. 2002; Soler et al. 2003), in which the effect of warming rate on the survival or metabolic activity of biological specimens (fetal pancreases, spermatosoa) depending on the cooling rate has been studied. In all these works authors used a constant warming rate (low or high) in a temperature range between the temperature of storage and melting. However, up till now there have been no studies on variable warming rate, which value is depended on the phase transformation processes. As a result of our investigation, the general principles for the selection of the numerical values of cooling and warming rates can be formulated: the high rate (20 °C/min) should be used on the stages of the eutectic crystallization and recrystallization, and the low rate (1 °C/min) should be used on the stages of the eutectic melting.

Thus, the advantage of using the controlled warming rates consists in providing a better recurrence of the results due to the exclusion of random errors that are inevitable with the manual sample thawing on a water bath. Thawing in the programmable freezer with controlled warming rates guarantees the high cell-based product quality due to the precise control of the thermal history of samples and the minimization of the cell contact with the contamination agents.

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