Open Access

Jennifer Contreras Lopez*, Lothar Lauterböck and Birgit Glasmacher

Determination of the membrane hydraulic permeability of MSCs

DOI 10.1515/cdbme-2016-0072

Abstract: A successful cryopreservation is based on knowledge of the optimal cooling rate. So far, this is often determined by way of complex parameter studies. Alternatively, the identification of cell specific characteristics, such as osmotic behaviour, membrane hydraulic permeability and activation energy could be used to calculate the optimal cooling rate. These parameters should be determined for supra-zero and sub-zero temperatures. In this study cryomicroscopy was used. Mesenchymal stromal cells (MSCs) from bone marrow were analysed. The determined membrane hydraulic permeability for sub-zero temperatures is significantly lower than that for supra-zero temperatures. On the contrary the activation energy is significantly higher in the presence of ice. The addition of a cryoprotective agent (CPA) such as dimethyl sulfoxid (DMSO) shows an additional influence on the characteristics of the membrane of the cell. The optimal cooling rate was determined with these parameters. For cryopreservation without DMSO the optimal cooling rate was found to be 12.82 K/min. If the MSCs were frozen with 5% (v/v) DMSO the optimal cooling rate is 16.25 K/min.

Keywords: cryopreservation; optimal cooling rate; membrane hydraulic permeability; mesenchymal stromal cells.

1 Introduction

Regenerative medicine is one of the areas with the most dynamic growth. The main tool is vital cells, especially stem cells, which have enormous potential due to their multifunctional characteristics [1]. A continuous provision with viable and functioning cells is required in order to get a successful application [2]. Therefore, the feasibility of long-term cell storage plays an important role in regenerative medicine [3]. Currently, cryopreservation is the only method to store biological material and vital cells permanently without damage [4]. A successful cryopreservation is based on the knowledge of the correct CPA and the optimal cooling rate. So far, the optimal cooling rate is determined often experimentally by complex, time-consuming and costly parameter studies [5]. In order to avoid these, the optimal cooling rate can also be calculated via cell specific biophysical parameters. Common parameters to be ascertained in this respect are the osmotic behaviour, the membrane hydraulic permeability and the activation energy [6, 7].

In this study the biophysical parameters of MSCs (from bone marrow) are examined by cryomicroscopy. By using the cryomicroscope it is not only possible to observe cell volume changes at different salt concentrations but also in connection with supra-zero and sub-zero temperatures. By measurement of the volume change and subsequent application of theoretical models the biophysical parameters can be determined. With knowledge of these parameters the optimal cooling rate can be calculated deduced.

2 Material and methods

2.1 Mesenchymal stromal cells

The MSCs were initially isolated from the bone marrow of the common marmoset monkey by the group of T. Müller [8]. The MSCs were cultured in Dulbecco's modified Eagle's medium (DMEM) with 15% fetal bovine serum (FBS), 0.1% ascorbic acid and 1% penicillin/streptomycin (Biochrom GmbH, Germany) until reaching 80% confluence in a CO_2 incubator at 37°C and 5% CO_2. For the counting of cells the cell analyser Vi-Cell™ XR (Beckman Coulter GmbH, Krefeld, Germany) was used.

2.2 Solutions

For the examination of osmotic behaviour, Hydroxyethyl piperanzine-ethanesulfonic (HEPES) buffered solutions

***Corresponding author: Jennifer Contreras Lopez,** Institute for Multiphase Process, E-mail: jennifer.contreras-lopez@gmx.de
Lothar Lauterböck and Birgit Glasmacher: Institute for Multiphase Process, Callinstraße 36, 30167 Hannover, E-mail: lauterboeck@imp.uni-hannover.de (L. Lauterböck); glasmacher@imp.uni-hannover.de (B. Glasmacher)

with salt concentrations of 240, 260, 300, 400, 500 and 600 mosm/kg were used. The cryopreservation of the MSCs had been carried out with and without 5% (v/v) DMSO.

2.3 Cryomicroscopy

All tests were executed under the microscope *Axio Imager* (Carl-Zeiss, Germany) and images were recorded by using the digital video camera (Retiga ECi Past 1394, QImaging). The cooling profile was set with the temperature-controlled chamber Linkam *FDCS-196*.

First, the osmotic behaviour at different constant temperatures of 0, 4, 15, 22 and 37°C was observed under the cryomicroscope. For this purpose, 10^6 cells were prepared in an Eppendorf tube with 200 µl medium. 20 µl of the cell suspension is mixed with 50–75 µl MSC-medium on a Poly-L-Lysin (PLL) plate which is located in a quartz crucible (Resultec, Germany). After the appropriate temperature has been reached, the MSC-medium was removed and 50–75 µl HEPES solution was added. After adding the corresponding HEPES solution, the change in volume is determined over time.

By freezing investigation the change in volume depending on the temperature has been determined. For this, the experimentally identified-cooling rate of 7.5 K/min to −30°C and 3 K/min to −80°C was used under the cryomicroscope. In each Eppendorf tube 10^6 cells were prepared in 50 µl medium for the experiments. 50 µl of the respective cryo solution is slowly added (with or without DMSO) to the cell suspension and resuspended. 5 µl of this suspension is now pipetted into the middle of the quartz crucible and covered with a small sterile cover slip.

The evaluation of all cryomicroscope recordings was carried out using the software *ImageJ fiji*. For the osmotic experiments the volume of 20 cells per frame is determined and formed the arithmetic mean. The evaluation of the freezing tests is done with 12–14 cells per frame from the moment of entering nucleation.

2.4 Estimation of the membrane hydraulic permeability

The transportation of water and CPAs across the cell membrane together with the change in volume of the cell has been investigated by means of theoretical models.

The membrane hydraulic permeability at supra-zero temperatures is identified in the presence as well as in the absence of CPAs by two coupled differential equations. They describe the change in volume and in solution movement across the membrane as a function of time (dV/dt). The water flow of the cell is given by [6]:

$$\frac{dV}{dt} = Lp \cdot A \cdot R \cdot T \cdot \left(M^e - M^i\right) \tag{1}$$

Lp: Membrane hydraulic permeability, R: Universal gas constant, T: Temperature, M: Osmolality, M^e–M^i Osmotical gradient between extra- und intracellular solution.

The membrane hydraulic permeability at sub-zero temperatures is described by an extended water transport model. This describes the change in volume during freezing of the cell as a function of the cell dimension and the membrane permeability parameter. To integrate the effects of the CPA, further modifications of the model were carried out. Frequently used in this regard is the 2-parameter-model of Mazur, which is used in its simplified form as follows [9]:

$$\frac{dV}{dT} = \frac{Lp \cdot A \cdot R \cdot T}{v_w \cdot B} \left[\ln\left(\frac{(V_0 - V_b - n_{cpa} \cdot v_{cpa})}{(V_0 - V_b - n_{cpa} \cdot v_{cpa}) + v_w \cdot (v_s \cdot n_s + n_{cpa})}\right) - \frac{\Delta H_f}{R} \cdot \left(\frac{1}{T_R} - \frac{1}{T}\right)\right] \tag{2}$$

The meaning of the relevant parameters and the values being used for this work are shown in the following table (see Table 1).

Table 1:

Symbol	Description	Value	SI unit
A	Membrane surface area of the cell	377.44	m^2
B	Cooling rate	7.5	K/min
ELp	Activation energy		J/mol
ΔHf	Heat of fusion of water	6010.57	J/mol
Lp	Membrane hydraulic permeability		m/sPa
Lpg	Permeability of the membrane to water at T_R		m/sPa
n_{cpa}	Number of moles of CPA (DMSO) in the cell	3.19×10^{-13}	g/mol
n_s	Number of moles of solutes in the cell	1.49×10^{-13}	mol
R	Universal gas constant	8.314	J/Kmol
T	Temperature		K
T_R	Reference temperature	273.15	K
V	Volume		m^3
V_0	Isotonic volume	689.52	m^3
V_b	Osmotically inactive volume	191.78	m^3
v_{cpa}	Molar volume of CPA (DMSO)	7.1×10^{-5}	mol
v_s	Dissociation constant of salt	2	m^3/mol
v_w	Molar volume of water	1.8×10^{-5}	m^3/mol

2.5 Arrhenius relation

The water transport across the cell membrane depends on temperature. A measurement for this dependence can be described by the activation energy. The activation energy can be ascertained by using the Arrhenius-correlation and the Arrhenius-graph. The connection between membrane hydraulic permeability and temperature can be determined by using the Arrhenius equation [10]:

$$L_p = L_{pg} \cdot e^{\left[-\frac{E_{Lp}}{R}\left(\frac{1}{T}-\frac{1}{T_R}\right)\right]} \quad (3)$$

L_{pg}: Permeability of the membrane to water at T_R, T_R: Reference temperature (273.15 K), E_{LP}: Activation energy, R: Universal gas constant.

2.6 Calculation of the optimal cooling rate

Knowing the biophysical parameters, the optimal cooling rate is determined by the following equation [11]:

$$B_{opt} = 1009.5 \cdot e^{-0{,}0546 \cdot E_{Lp}} \cdot L_{pg} \cdot \frac{A}{V_w} \quad (4)$$

E_{LP}: Activation energy for the permeation process, L_{pg}: Permeability of the membrane to water at T_R, A: Membrane surface area of the cell, V_w: Osmotically active volume.

3 Results

3.1 Boyle van't Hoff behaviour

In order to determine the isosmotic volume as well as the osmotic inactive volume of the MSCs the cell volume has been determined by cryomicroscopy. The cell volume in isotonic medium V_0 was determined to be 689.2 ± 32.8 µm^3. Figure 1 shows the Boyle van't Hoff plot of the cell volume data. The cells behave as linear osmometers in the investigated osmotic range (200–600 mosm/kg). The osmotic inactive volume V_b, that was obtained from extrapolation to infinite osmolality was to be 0.275 of V_0 (189.6 ± 9.02 µm^3). So the osmotic active volume was determined to be 497.74 µm^3.

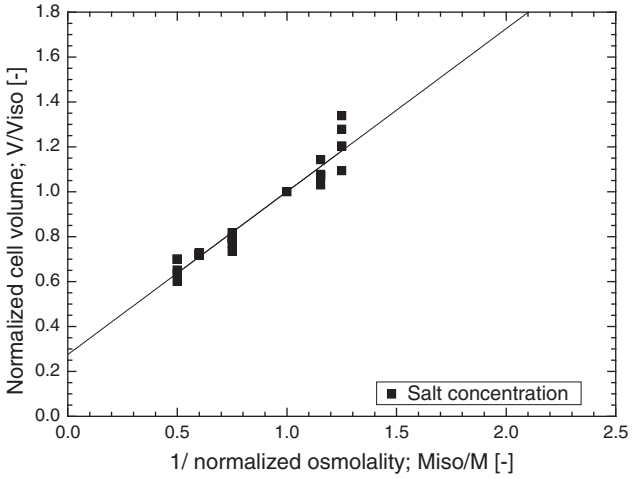

Figure 1: Boyle van't Hoff relationship of MSCs.

Table 2:

Concentration of salt	Lp [m/sPa]	Lpg [m/sPa]	ELp [J/mol]
240	1.29×10^{-13} to 3.10×10^{-14}	0.195	5.975
260	6.48×10^{-14} to 3.61×10^{-14}	0.221	2.71
400	3.92×10^{-14} to 1.72×10^{-14}	0.102	3.623
500	2.92×10^{-14} to 1.21×10^{-14}	0.102	4.084
600	1.79×10^{-14} to 1.12×10^{-14}	0.071	2.03

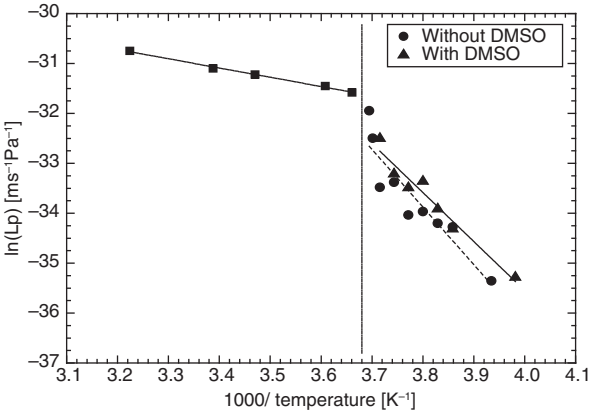

Figure 2: Arrhenius plot of supra-zero and sub-zero membrane hydraulic parameters.

3.2 Supra-zero membrane hydraulic parameters of MSCs

For the supra-zero membrane hydraulic parameters the following results have been determined (see Table 2 and Figure 2). On average, the Lpg is 0.13 µm/min and the corresponding activation energy is 3.68 kcal/mol.

3.3 Sub-zero membrane hydraulic parameters of MSCs

The results of membrane characteristics for sub-zero temperatures in contrast to the previous results show that

the membrane hydraulic permeability is lower and the activation energy is higher (see Figure 2). With DMSO, the permeability of the membrane to water is 0.06 µm/min atm and the corresponding calculated activation energy for water is 19.50 kcal/mol. Without DMSO, the permeability of the membrane to water is 0.059 µm/min atm and the activation energy is 22.94 kcal/mol.

3.4 Optimal cooling rate

Based on the previously calculated biophysical parameters the optimal cooling rate for the MSCs has been calculated by equation 4. For cryopreservation without DMSO, the optimal cooling rate results in 12.82 K/min. If the MSCs are frozen with 5% DMSO (v/v), the optimal cooling rate results in 16.25 K/min.

The survival rates of MSCs which have been frozen by using the mathematically calculated cooling rate in comparison to the ones which were frozen by using the experimental two-step cooling rate show similar results (see Figure 3). The recultivation efficiency shows an average of $78.0 \pm 9.8\%$. In contrast the survival rate of MSCs frozen without DMSO showed a significant difference compered to all other groups ($p < 0.001$). Here the survival rate is only $27.4 \pm 7.9\%$.

4 Conclusion

For a successful cryopreservation with high survival rates, the knowledge of cell-specific parameters is essential. Therefore, in this study the biophysical parameters of MSCs from bone marrow have been identified. By using cryomicroscopy, the osmotic behavior, the membrane permeability and the activation energy were examined. The decisive factor here is to examine the different behaviors at supra-zero and sub-zero temperatures. With knowledge of these parameters, the optimal cooling rate of MSCs could be finally determined and showed similar results to the experimental developed cooling rate.

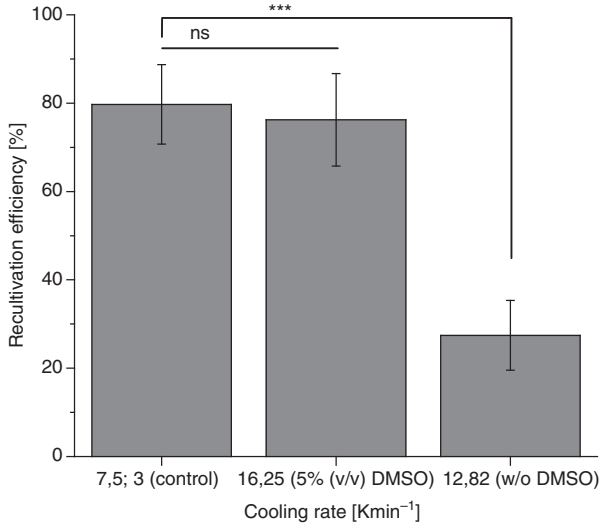

Figure 3: Measurement of recultivation efficiency. ns: not significant, ***: $p < 0.001$.

Author's Statement

Research funding: The author state no funding involved. Conflict of interest: Authors state no conflict of interest. Material and Methods: Informed consent: Informed consent is not applicable. Ethical approval: The conducted research is not related to either human or animal use.

References

[1] Marquez-Curtis LA, Janowska-Wieczorek A, McGann LE, Elliott JAW. Mesenchymal stromal cells derived from various tissues: Biological, clinical and cryopreservation aspects. Cryobiology. 2015;71:181–97.

[2] Hunt CJ. Cryopreservation of human stem cells for clinical application: a review. Transfusion medicine and hemotherapy: offizielles Organ der Deutschen Gesellschaft fur Transfusionsmedizin und Immunhamatologie, 2011;38:107–23.

[3] Davies OG, Smith AJ, Cooper PR, Shelton RM, Scheven BA. The effects of cryopreservation on cells isolated from adipose, bone marrow and dental pulp tissues. Cryobiology. 2014;69:342–7.

[4] Vian AM, Higgins AZ. Membrane permeability of the human granulocyte to water, dimethyl sulfoxide, glycerol, propylene glycol and ethylene glycol. Cryobiology. 2014;68:35–42.

[5] Bernemann I, Hoffmann N, Glasmacher B. Optimierung von Kryokonservierungsprotokollen: Systematische Parameteranalyse. Wissenschaft Kryotechnik/Kryokonservierung 2008.

[6] Akhoondi M, Oldenhof H, Stoll C, Sieme H, Wolkers WF. Membrane hydraulic permeability changes during cooling of mammalian cells. Biochimica et Biophysica Acta. 2011;1808:642–8.

[7] Katkov II. Challenge from the simple: Some caveats in linearization of the Boyle–van't Hoff and Arrhenius plots. Cryobiology. 2008;57:142–9.

[8] Bernemann I, Mueller T, Blasczyk R, Glasmacher B, Hofmann N. Colonization of collagen scaffolds by adipocytes derived from mesenchymal stem cells of the common marmoset monkey. Biochem Biophys Res Commun. 2011;411:317–22.

[9] Kleinhans FW. Membrane permeability modeling: Kedem-Katchalsky vs a two-parameter formalism. Cryobiology. 1998;37:271–89.

[10] Xu Y, Zhang L, Xu J, Wei Y, Xu X. Membrane permeability of the human pluripotent stem cells to Me2SO, glycerol and 1,2-propanediol. Arch Biochem Biophys. 2014;550–551: 67–76.

[11] Pinisetty D, Huang C, Dong Q, Tiersch TR, Devireddy RV. Subzero water permeability parameters and optimal freezing rates for sperm cells of the southern platyfish, Xiphophorus maculatus. Cryobiology. 2005;50:250–63.