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hMSCs from UCB: Isolation, Characterization and Determination of Osmotic Properties for Optimal Cryopreservation

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In tissue engineering, storing of biological material represents a fundamental step to bring cell-based medical devices to market on demand - Karlsson and Toner (2000) and more recently Fadda et al. (2009). Compared to other methods, freezing to cryogenic temperatures allows long shelf lives and genetic stability (Karlsson and Toner, 2000).
Unfortunately, cryopreserved cells are damaged by the cryopreservation process itself (Mazur, 2004). This loss (up to 50 %) can be tolerated for some cell lineages, but it's unacceptable for others, as the human Mesenchymal Stem Cells (hMSCs) from Umbilical Cord Blood (UCB), whose collection and isolation is known to be difficult (Bieback et al., 2004). In this case, an optimal cryopreservation protocol is mandatory. Due to the high number of trials actually required for experimental optimization, mathematical modelling is considered a practical solution. To this aim, the osmotic properties need to be first estimated in order to determine the volume of residual intra-cellular water left by osmosis to form lethal ice or glass.
In this work, the hMSCs from UCB of three different donors, after informed consent, have been isolated by a density gradient centrifugation method. The successful isolation has been verified through phenotypic cytofluorimetric analysis, and adipogenesis/osteogenesis capability differentiations. Osmotic properties, namely inactive cell volume, water and CPA (DMSO) permeabilities, have been determined by means of experimental runs carried out under hypertonic conditions (obtained with the addition of sucrose or DMSO), at three different temperatures. Cells volumes excursions have been measured by a potenziometric device (Coulter Counter) under equilibrium and dynamic conditions. Linear and non-linear regression analyses have been carried out to determine the adjustable parameters by means of the two parameters bi-compartimental model by Kleinahns (1998), as applied to a single-sized cell population (i.e. identical cells with size equal to the average).
It is found that, the inactive volume fraction of hMSC from UCB apparently changes (increase) when DMSO is used instead of sucrose, thus limiting cell volume excursion during swelling. It is hypothesized that, a cell volume control system is activated during swelling, probably due to the action of ion pumps.

1. Materials and Methods

1.1 Isolation and characterization of hMSC from UCB

15 UCB units were provided by the Banca del Cordone Ombelicale (Azienda Ospedaliera Brotzu, Cagliari). These units were processed within 15 h since birth time. Specifically, a density gradient method followed by adhesion was adopted in order to isolate the hMSCs, according to the protocol published by Bieback et al., (2004). hMSCs isolation was successful only for 3 UCB units (20 % success rate), thus confirming the difficulties of such operation.

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The successful isolation has been tested through phenotypic cytofluorimetric analysis, and adipogenesis and osteogenesis capability differentiation. Specifically, six antigenes expressions (CD 34-, 44+, 45-, 90+, 105+ from Millipore, and CD 73+ from BD Pharmingen) were assessed cytofluorimetrically (BD FACScan, BD Biosciences, Franklin Lakes, NJ, USA) as analysed by Kaluza Analysis software (Beckman Coulter, USA). In addition, adipogenesis and osteogenesis capability differentiation was verified by maintaining the cells in commercially available differentiating culture terrains (Stem Pro Adipogenesis and Osteogenesis Differentiation Kit, Gibco by Life Thechnologies). The accumulation of mineralized calcium phosphate was assessed by von Kossa staining, while lipid-rich vacuoles accumulation stained with Oil Red O (Sigma-Aldrich) was used to verify adipogenesis.

1.2 Osmotic Runs

Isotonic hMSCs were equilibrated with hypertonic solutions obtained by adding sucrose and/or DMSO (Sigma-Aldrich) to isotonic PBS (300 mOsm/L), at three system temperatures (17, 27 and 37 °C). Osmolality was measured by means of a freezing point depression osmometer (Advanced Micro Osmometer Model 3300, Advanced Instruments, Norwood, MA). Isotonic cells and hypertonic solutions were contacted in such relative amounts between the two phases (from 2,000 to 10,000 cells/mL) that, during any single run, the composition of the extra-cellular compartment (i.e. the suspending solution) did not change due to the osmotic response of the cells (the intra-cellular compartment). Temperature was kept constant during each single run.

Apparatus and operating conditions

Cells volume was measured by means of the Coulter Counter Multisizer 4 (Beckman Coulter). Transient as well as equilibrium runs were performed, thus measuring dynamically the osmotic response, or measuring the cells volumes only at final, equilibrium time (5 min), as determined by preliminary runs.
Specifically, equilibrium runs were all performed at T = 27 °C with hypertonic solutions obtained by adding only sucrose to PBS isotonic solutions (thus reaching 400, 500, 560, 600 and 900 mOsm/L).
Dynamic runs were performed for all the three temperatures by equilibrating cells with a single hypertonic solution osmolality (obtained by adding only sucrose to PBS isotonic solutions, thus reaching 600 mOsm/L), or by adding only DMSO to PBS isotonic solutions (thus reaching 2,000 mOsm/L). During the osmotic runs, system temperature was controlled (± 0.2 °C) by circulating water-NaCl bath (2 % wt/vol) through a jacketed and perfectly mixed beaker, where isotonic cells were injected to contact with the hypertonic solutions.
In addition, only at T = 27 °C dynamic runs were performed with hypertonic solutions obtained by adding only DMSO to PBS isotonic solutions (thus reaching 1,000 and 1,500 mOsm/L) and by adding DMSO and sucrose to PBS isotonic solutions (thus reaching 2,365 mOsm/L, with 65 mOsm/L due to sucrose and 2,000 mOsm/L to DMSO).
The experimental runs described above were performed for each single donor, separately.

Data treatment

The osmotic response of cells, measured potenziometrically by means of the Coulter Counter, allows one to follow a high number of cells, as in cytofluorimetry but in contrast with the use of image analysis (i.e. microscopy). Indeed, with image analysis only the volume of the limited number of cells focused under the microscope may be determined, while, by means of the Coulter Counter, the entire distribution of volumes inside a cell population (naturally expected, according to the corresponding cell life-cycle) may be measured. On the other hand, the potenziometric device does not discriminate single cells by debris or cells agglomerates. For this reason, the measured cells volume distributions were filtered from the data originally measured by the Coulter Counter. Basically, debris (characterized by small volumes) and agglomerates (characterized by large volumes) were discharged by eliminating all the measured volumes outside a dynamic volumic range, identified from the mimimum and maximum volumes of the cells distributions.
In any size distribution, the differences on osmotic behaviour among differently sized class of cells (in terms of the dynamics of the cell volume of any class normalised to the corresponding isotonic value) were among the errors of the experimental measurements. For this reason, the size distribution measured by the Coulter Counter were averaged by determining the medium cells volume (as the first moment of the size distribution).
According to the experimental runs, the data treatment was performed for each single donor, separately. Then data were pooled among the three donors, by calculating the arithmetic means of the average cells volume measured from each single donor.

Modeling

In the framework of the mathematical modelling of cell osmotic response related to cryopreservation, the behaviour of a single cell is usually described through the ‘‘saltwater sack’’ model. Basically, the cell is seen as a spherical drop of a salty (NaCL) aqueous, ideal solution representing the cytoplasm, where proteins,

organelles, and other macromolecules are suspended. For osmosis all these materials suspended in the cytoplasm are actually inactive. As such, they are lumped together in the inactive cell fraction υ_b which remains constant during osmotic response. In this regard, differently sized cells belonging to the same lineage are assumed to be constituted by the same inactive cell fraction υ_b, which is then considered a characteristic feature of the cellular lineage.

This cell structure is separated from the exterior environment by an oil semi-permeable membrane, which rules the movement of any molecule between the intra- and the extra-cellular compartments composing the system. Cell membrane is characterized by a low resistance to water transport, whilst the permeability to a specific solute depends on its size, electric charge and the features of hydrogen bonding. Traditionally, in osmotic investigations related to cryopreservation, sucrose and NaCl are assumed to be not permeant through the cells membrane. Thus, the content of NaCl does not change in both the intra- and the extra-cellular compartments. On the contrary, a permeant CPA like DMSO may be transferred from the external medium to the interior of the cells and vice versa (as the water), so that its content in both compartments may change.

In this work, the dynamic response of a single, spherical and isotonic cell during the osmotic runs is described on the basis of the following 2-parameters model by Kleinahns (1998):

$$\frac{dV_w}{dt} = -L_P RTA\left[\left(M^{ext}_{NaCl+Sucrose} - M^{\text{int}}_{NaCl}\right) + \left(M^{ext}_{DMSO} - M^{\text{int}}_{DMSO}\right)\right] \qquad V_w = V_w^{iso} \quad @ \quad t = 0 \tag{1}$$

$$\frac{dV_{DMSO}}{dt} = \tilde{\upsilon}_{DMSO} P_{DMSO} A\left(M^{ext}_{DMSO} - M^{\text{int}}_{DMSO}\right) \qquad V_{CPA} = 0 \quad @ \quad t = 0 \tag{2}$$

Here V_w and V_{DMSO} represent the water and DMSO volumic contributions to cell volume V_{cell}; R is the universal gas constant; T is the system temperature; A is the cell membrane area (assumed to change spherically with V_{cell}); $\tilde{\upsilon}_{DMSO}$ is the DMSO molar volume (equal to 7.1 10^{-5} m^3/mol).

The water L_P and solutes P_{DMSO} permeabilities to membrane are considered characteristic features of a cellular lineage just like the inactive cell fraction υ_b, with an Arrhenious-like dependence from temperature ($L_p = L_p^0 \exp\left(-\frac{E_w}{RT}\right)$ and $P_{DMSO} = P^0_{DMSO} \exp\left(-\frac{E_{DMSO}}{RT}\right)$). L_p and P_{DMSO} represent the two adjustable parameters that gives the name to the model proposed by Kleinahns (1998). On the basis of the ''saltwater sack'' model, V_{cell} is equal to $\left(V_w + V_{DMSO} + V_{NaCl} + V_b\right)$ with $V_b = \upsilon_b V^{iso}_{cell}$ representing the inactive cell volume and V_{NaCl} the sodium chloride contribution. The latter one is negligible, but both remain constant during any osmotic run.

According to Eq(1) and (2), intracellular volumes of water V_w and DMSO V_{DMSO} change osmotically (and with them V_{cell} change as well), depending on the driving forces of solutes concentrations between intra- and extra-cellular compartments, expressed in terms of osmolalities M of not permeant (as NaCL and sucrose), and permeant (as DMSO) solutes. Specifically, starting from the isotonic condition (i.e. absence of intracellular DMSO), $M^{\text{int}}_{NaCl} = M^{iso}_{NaCl}\left(\frac{1-\upsilon_b}{\frac{V_{cell}}{V^{iso}_{cell}} - \upsilon_b - \frac{V_{DMSO}}{V^{iso}_{cell}}}\right)$ and $M^{\text{int}}_{DMSO} = \frac{1}{\tilde{\upsilon}_{DMSO}}\left(\frac{\frac{V_{DMSO}}{V^{iso}_{cell}}}{\frac{V_{cell}}{V^{iso}_{cell}} - \upsilon_b - \frac{V_{DMSO}}{V^{iso}_{cell}}}\right)$ represent the intracellular osmolalities that change during any osmotic run in order to reduce till zero the differences with the corresponding extra-cellular counterparts. In such a case, the osmotic equilibrium condition between the intra- and extra-cellular compartments is reached when cell volume and its DMSO contribution will attain the following values:

$$\frac{V_{cell}\big|_{equil}}{V^{iso}_{cell}} = \upsilon_b + \left(1-\upsilon_b\right)\frac{M^{iso}_{NaCl}}{M^{ext}_{NaCl+Sucrose}}\left(1 + \tilde{\upsilon}_{DMSO} M^{ext}_{DMSO}\right) \tag{3}$$

$$\frac{V_{DMSO}\big|_{equil}}{V^{iso}_{cell}} = \tilde{\upsilon}_{DMSO}\frac{M^{ext}_{DMSO}}{1 + M^{ext}_{DMSO}}\left(\frac{V_{cell}\big|_{equil}}{V^{iso}_{cell}} - \upsilon_b\right) \tag{4}$$

In particular, Eq(3) states that, in absence of CPA, a plot of $\frac{V_{cell}\big|_{equil}}{V^{iso}_{cell}}$ vs $\frac{M^{iso}_{NaCl}}{M^{ext}_{NaCl+Sucrose}}$ is a line whose intercept

is υ_b and slope $(1-\upsilon_b)$, that is a line between points $\left(\frac{M_{NaCl}^{iso}}{M_{NaCl+Sucrose}^{ext}}=0;\frac{V_{cell}\big|_{equil}}{V_{cell}^{iso}}=\upsilon_b\right)$ and $\left(\frac{M_{NaCl}^{iso}}{M_{NaCl+Sucrose}^{ext}}=1;\frac{V_{cell}\big|_{equil}}{V_{cell}^{iso}}=1\right)$. This represents the so called Boyle Van't Hoff plot. It is worth noting that, as M_{DMSO}^{ext} increases from zero, the Boyle Van't Hoff plot is always a line with the same intercept, but increased slope, so that it will go through the point $\left(\frac{M_{NaCl}^{iso}}{M_{NaCl+Sucrose}^{ext}}=1;\frac{V_{cell}\big|_{equil}}{V_{cell}^{iso}}=1+\tilde{\upsilon}_{DMSO}M_{DMSO}^{ext}(1-\upsilon_b)\right)$. In other words, adding DMSO to isotonic PBS will result in an increased $V_{cell}\big|_{equil}$ with respect to V_{cell}^{iso}, i.e. a swelling cell.

2. Results and discussion

For the sake of brevity, the characterization of isolated cells through phenotypic cytofluorimetric analysis, and adipogenesis and osteogenesis capability differentiation are not reported in this paper.

According to the model Eq(1) and (2), an ideal fitting procedure to determine υ_b, L_p and P_{DMSO} consists on the following sequential steps: first, determine υ_b from the Boyle Van't Hoff plot resulting from the equilibrium runs of isotonic cells with different hypertonic solutions (obtained adding only sucrose to PBS isotonic solutions, at a given temperature); then, with υ_b kept constant at the value just set, determine L_p and its Arrhenius dependence from the dynamic runs of isotonic cells with a single hypertonic solution osmolality (obtained by adding only sucrose to PBS isotonic solutions, carried out at the three temperatures levels investigated); finally, with υ_b and L_p now fixed, determine P_{DMSO} and its Arrhenius dependence from the dynamic runs of isotonic cells with a single hypertonic solution osmolality (obtained by adding only DMSO to PBS isotonic solutions, carried out at the three temperatures levels investigated).

Following this ideal procedure, In Figure 1a the Boyle Van't Hoff plot for the hMSCs from UCB is reported. The good linear regression analysis of experimental data through Eq(3) gives an estimate of υ_b equal to 0.19. The model correctly follows the osmotic response of a shrinking cell in hypertonic solutions of not permeant solutes. This linearity is a feature usually attributed to a "perfect osmometer" behaviour. A successful non-linear regression analysis is obtained for determining L_p as well ($L_p^0=154.5\frac{\mu m}{Pa\ s}; E_w=51.84\frac{kJ}{mol}$), as shown in Figure 1b. Here, the dynamics of the normalised cell volume to its isotonic value, at three different temperatures but constant hypertonic osmolality, is given.

Since a not-permeant solute is used, the normalised cell volume decreases in time till reaching a constant value, regardless of the system temperature - as stated by Eq(3). However, at the final step of the ideal fitting procedure, the contact of isotonic cells with a hypertonic solution of a permeant solute is not correctly followed by the model.

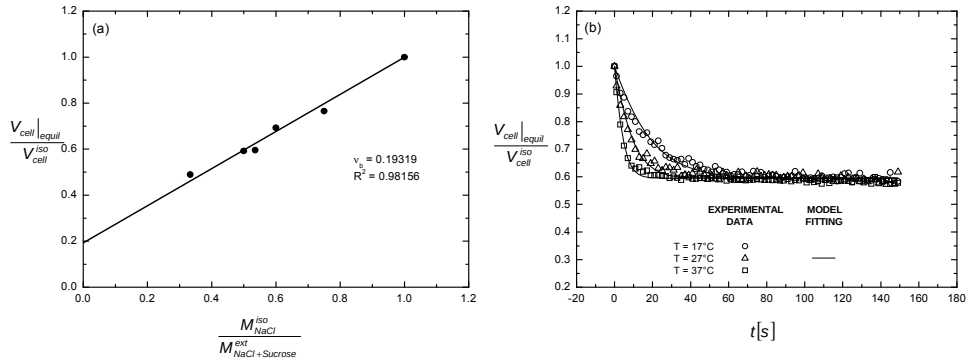

Figure 1: Boyle Van't Hoff plot (a); Dynamic shrinkage of isotonic cells to hypertonic (not permeant) solutes (b)

This is shown in Figure 2a, where the dynamics of the normalised cell volume is given at the same three different temperatures used for Figure 1a. In such conditions, the isotonic cell first shrinks then swells, due to the relatively high permeability of water in comparison with DMSO permeability. This is a well-known

behaviour as reported in the literature for other cell lineages. It is here shown for the hMSCs from UCB at all the three temperatures. The model fitting corresponding to the final step of the ideal procedure is reported as dot lines in Figure 2a.

Even if, during shrinkage, the initial slope and its temperature dependence (related to L_p) are somehow followed by the model, during the swelling phase it fails completely. Basically, in these new data the normalised cell volume is constantly under 1, while, according to Eq(3), adding DMSO to isotonic PBS should result in an increased $V_{cell}|_{equil}$ with respect to V_{cell}^{iso}, i.e. a swelling cell. In addition, the obtained values of P_{DMSO} are not correctly interpreted by the Arrhenius temperature dependence (not shown here). It appears that, in order to limit volumic excursions, a higher υ_b should now be used in the model instead of the one previously determined when using a not-permeant solute (sucrose). This hypothesis is supported, by the model fitting of only the data of the first 30 s (cf. dash lines in Figures 2a). The match is now improved initially, but still lacks, especially at the final, equilibrium time. In other words, υ_b should change when using not-permeant and permeant solutes.

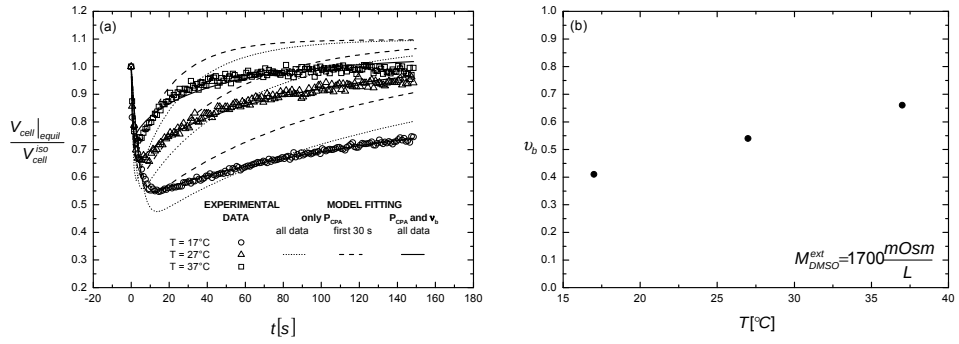

Figure 2: Dynamic shrink-swell behaviour of isotonic cells to hypertonic (permeant DMSO) solute (a); Temperature dependence of υ_b *when determined from contacting isotonic cells to hypertonic (permeant DMSO) solute at constant concentrations (b)*

For this reason, the hMSCs from UCB cannot be called a “perfect osmometer”, and, accordingly, the ideal fitting procedure given above cannot be adopted to determine their osmotic parameters as classically adopting Eq(1) - (4).

Therefore, the data shown in Figure 2a have been fitted by the model by adjusting both P_{DMSO} and υ_b (cf. bold solid lines), at the three system temperatures investigated. Now a perfect match is obtained and an Arrhenius temperature dependence results for P_{DMSO} ($P_{DMSO}^0 = 3.2\times10^8 \frac{\mu m}{s}; E_{DMSO} = 56.55 \frac{kJ}{mol}$), not shown for brevity. However, υ_b changes linearly with temperature as shown in Figure 2b.

Based on this, a model validation has been performed by full prediction of experimental data measured at the highest DMSO concentration used in this work, i.e. outside the range used so far to fit the data, but at T = 27 °C. A reasonable matching between model results and experimental data is obtained as shown in Figure 3a, where the dynamic response of the normalised cell volume is reported. This confirms the values obtained for the adjusted parameters related to transport (L_p and P_{DMSO}), and their Arrhenius dependence, which are among the typical values available in the literature for different cell lineages - Hunt et al. (2003) and more recently Vian and Higgins (2014). To the best of authors’ knowledge, these osmotic transport parameters have never been published before for the hMSCs from UCB. The only osmotic parameter reported in the literature for this cell lineage is υ_b. Specifically, Parekkadan et al. (2007) reported a value equal to 0.6 as determined from a Boyle Van’t Hoff plot obtained contacting isotonic cells with hypertonic solutions of a not-permeant solute (mannose). This value is very different from the corresponding value (almost 0.2) here reported in Figure 1a, where sucrose was used. On the other hand, it should be noted that Parekkadan et al. (2007) adopted the image analysis technique to measure cell volumes, while in this work a potenziometric device was used.

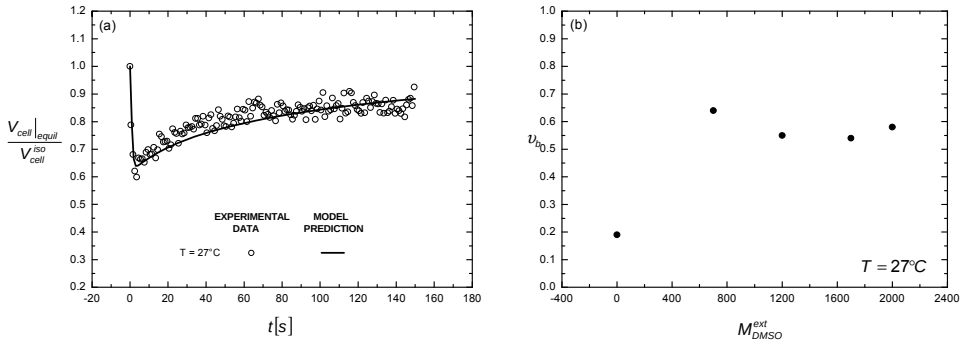

Figure 3: Dynamic shrink-swell behaviour of isotonic cells to hypertonic (permeant DMSO) solute (a); Dependence of υ_b *when determined from contacting isotonic cells to hypertonic (permeant DMSO) solute at constant temperature but different concentrations (b)*

From the kinetics analysis carried out in this work, at constant temperature a dependence of υ_b by DMSO concentration results, as shown in Figure 3b. It appears that, starting from the lowest value when CPA is absent, υ_b increases abruptly to an almost constant value around 0.6, valid for any concentration. On the basis of Figure 2b, this plateau of υ_b in the presence of DMSO appears to depend linearly on temperature. On the other hand, the rational fitting procedure conducted in this work suggests that, at least during shrinkage, the model does not suffer this DMSO effect and matching with experimental data is acceptable.

3. Conclusions

The hMSCs from UCB were successfully isolated and characterised. The kinetic investigation developed in this work shows that these cells do not behave as a perfect osmometer, i.e. an apparent variation of the inactive volumic fraction in the presence of a permeant solute as DMSO results. It may be speculated that, in the presence of DMSO, a regulatory volume system is activated for the cell, albeit only during swelling. On the other hand, this control system is presumably related to the action of ion pumps, which are well known in the literature for conditioning the cell to return to isotonic size, thus contrasting the lethal effect produced by osmosis. This ion pump action should increase with temperature.

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References

Bieback K., Kern S., Kluter H., Eichler H., 2004, Critical Parameters for the Isolation of Mesenchymal Stem Cells from Umbilical Cord Blood, Stem Cells, 22, 625–634.

Fadda S., Cincotti A., Cao G., 2009, A population balance approach for the description of water osmosis and intracellular ice formation during cryopreservation, Chemical Engineering Transaction, 17, 1125-1130.

Hunt C. J., Armitage S. E., and Pegg D. E., 2003, Cryopreservation of Umbilical Cord Blood: 1. Osmotically Inactive Volume, Hydraulic Conductivity and Permeability of $CD34^+$ Cells to Dimethyl Sulphoxide, Cryobiology, 46, 61-75.

Karlsson J.O.M., Toner M., 2000, Principles of Tissue Engineering. Academic Press, San Diego, USA.

Kleinhans F.W., 1998, Membrane Permeability Modeling: Kedem-Katchalsky vs a Two Parameter Formalism, Cryobiology, 37, 271-289.

Mazur P., 2004, Life in Frozen State. CRC Press, London, UK.

Parekkadan B., Sethu P. Van Poll D., Yarmush M.L., Toner M., 2007, Osmotic selection of human mesenchymal stem/progenitor cells from umbilical cord blood, Tissue Engineering, 13, 2465-2473.

Vian A. X., Higgins A. Z., 2014, Membrane Permeability of the Human Granulocyte to Water, Dimethyl Sulfoxide, Glycerol, Propylene Glycol and Ethylene Glycol, Cryobiology, 68, 35-42.