CryoLetters **39** (6), 380-385 (2018)

THEORETICAL ESTIMATION OF THE OPTIMUM COOLING RATE OF A CELL SUSPENSION AT LINEAR FREEZING MODES BASED ON A TWO FACTOR THEORY OF CRYODAMAGE

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Abstract

BACKGROUND: According to the two-factor theory cryodamage arises from intracellular crystallization and solution effects due to freeze concentration. **OBJECTIVE:** The study aims to evaluate the contribution of two types of cryodamages that are related to extra- and intracellular crystallization. **METHODS:** The probability of intracellular crystal formation during cooling of cell suspension in cryoprotective solution has been determined based on general thermodynamics theory. **RESULTS:** According to the obtained correlations and taking into account of the individual characteristics of yeast cells and murine enterocytes, the optimal cooling rates during freezing of these cells in cryoprotectant solutions were determined. **CONCLUSION:** The proposed algorithm for the estimation of the optimal cooling rates at linear freezing mode of a particular cellular suspension can be utilized to develope methods for cryopreservation of different cell suspensions.

Keywords: cryodamage, cryopreservation, freeze concentration, intracellular crystallization, murine enterocytes, *Saccharomyces cerevisiae*.

INTRODUCTION

Long-term preservation of cells and tissues requires the development of freezing techniques that minimizes the cryodamage. The significant factors leading to cryodamage during freezing are formation and growth of ice crystals. Cell integrity during cryopreservation depends on the cooling rate and this dependence has been shown, both experimentally and theoretically, to have a dome-shape. The relation implies an existence of an optimal cooling rate, which is explained by the two-factor theory of cryo-damage developed by Mazur (11).

According to this theory, the first factor of cryodamage arises from crystallization of the extracellular environment and is caused by cellular dehydration and an increase in the concentration and ionic strength of extra- and intracellular solutions due to a partial solvent-to-ice transition (2). An increase in cooling rate diminishes the damage as a result of less dehydration and shorter exposure to damaging factors. The second factor is the formation of intracellular ice crystals, having the same effect as extracellular crystallization and destroying cellular membrane structures mechanically. At high cooling rates intracellular crystallization is more probable and is considered maximally disastrous for cells (8,9,12). Overcooling results in supersaturation of intracellular solutions, which is defined as a difference between the concentrations of intracellular and extracellular solutions that are being crystallized. The overcooling represents a thermodynamic driving force leading to both intracellular crystallization and cell dehydration. The optimal cooling rate for a cell suspension is the cooling rate at which overcooling of the intracellular solution does not result in ice crystal formation and yet is fast enough to prevent prolonged dehydration that would damage cells. At a given cooling rate an

oversaturation of the intracellular solution depends on the rate of mass exchange between the cell and its environment, i.e. the permeability of cell membrane to cryoprotectants and water molecules, as well as on the surface-to-volume ratio for a particular cell type.

To aid the optimization of cryopreservation protocols for different cell types, we set to theoretically estimate the optimal cooling rates for cells with different characteristics (such as enterocytes and yeasts), taking into account of the influence of both types of cryodamage as well as the behaviours of different cells.

THEORETICAL CONSIDERATION

To determine the cooling rates at which the intracellular crystallization would occur, it needs to establish how the probability of intracellular crystallization depends on supercooling of the intracellular solution. Thermodynamic theory of crystal formation in solutions and general theory of the activated processes (5) were applied to establish this dependence. According to the thermodynamics, a substance in a metastable state will eventually transition into a stable state. The transition takes place by spontaneous occurrence of nuclei (representing a new phase) due to fluctuations in a homogeneous environment. When the nuclei of a new phase appear, their size has to be large enough to compensate for the energy-disadvantageous effect of the emerging surface partition. There is a certain minimal critical size that a new phase germ should have in order to become a center of this phase, since for sizes smaller and larger than a critical size, one or another phase is stable. Due to the rapid decrease in the probability of fluctuations with increasing germ size, the beginning of the phase transition is determined by the probability of the emergence of the nuclei of these minimal required sizes.

It is convenient to choose Helmholtz free energy F, with independent variables T, V and μ, as a main thermodynamic parameter. The convenience of this parameter is due to the fact that T and μ have the same value in either phase, whereas pressure is different and encompasses the surface effects. The average time $\langle t \rangle_i$ required for an ice crystal formation in a supersaturated aqueous solution at a constant temperature T, as well as for any isothermal process of the activation type, is determined by the expression

$$\langle t \rangle_i = C \exp \frac{\Delta F}{kT} \qquad [1]$$

where ΔF is the change in the free energy of the supersaturated solution during formation of an ice crystal; k is the Boltzmann's constant; T is absolute temperature (5).

During the formation of an ice crystal containing N_i number of water molecules, the change in the free energy of the thermodynamic system under consideration is equal to

$$\Delta F = N_i(\mu_i - \mu_w) - V_i(P_i - P_w) + \sigma S_i \qquad [2]$$

where μ_i and μ_w are the chemical potentials assigned to a water molecule in the crystalline and liquid phases, respectively; V_i is the volume of the ice crystal formed; P_i and P_w are the hydrostatic pressure inside the ice crystal and in the surrounding solution, respectively; σ is the surface tension coefficient of a solid-liquid interface; S_i is the surface area of the ice crystal.

Since ice crystals do not contain molecules of the substance dissolved in an aqueous solution, the chemical potential of water in ice depends only on the pressure P_i in the crystal and the solution temperature T: $\mu_i=\mu_i(P_i,T)$. The chemical potential attributed to a water molecule in the aqueous solution surrounding the crystal depends on the hydrostatic pressure P_w, temperature T and the concentration of the dissolved substance c_e: $\mu_w=\mu_w(P_w,T,c_e)$. Taylor series expansion of the functions $\mu_i=\mu_i(P_i,T)$ and $\mu_w=\mu_w(P_w,T,c_e)$ around state $P_w,T,\tilde{c}_e$ in a linear approximation *gi*ves

$$\mu_i(P_i,T) = \mu_i(P_w,T) + \left(\frac{\partial \mu_i}{\partial P}\right)_{P=P_w,T} (P_i - P_w) \qquad [3]$$

$$\mu_w(P_w,T,c_e) = \mu_w(P_w,T,\tilde{c}_e) + \left(\frac{\partial \mu_w}{\partial c_e}\right)_{P=P_w,T,c_e=\tilde{c}_e} (c_e - \tilde{c}_e) \qquad [4]$$

Assuming that the crystal's shape is spherical, we obtain

$$V_i = N_i \upsilon_i = \frac{4}{3}\pi R_i^3 \quad \text{and} \quad S_i = 4\pi R_i^2 \qquad [5]$$

where R_i is the ice crystal radius; υ_i is the volume of a water molecule in the crystalline phase. Using the condition of mechanical equilibrium between the spherical ice crystal and the surrounding solution $P_i - P_w = \frac{2\sigma}{R_i}$ (Laplace equation) and $\frac{\partial \mu_i}{\partial P} = \upsilon_i$ (the thermodynamic equation) instead [3] we obtain

$$\mu_i(P_i,T) = \mu_i(P_w,T) + \frac{2\sigma\upsilon_i}{R_i} \qquad [6]$$

Assuming that c_e is the number of moles of the dissolved substance per unit volume of an aqueous solution, and using the expression for the chemical potential of water in an ideal solution $\mu_w = -RT\upsilon_w c_e$, where R is the universal gas constant; υ_w is the volume of a water molecule in the liquid phase, instead [4] we get

$$\mu_w(P_w,T,c_e) = \mu_w(P_w,T,\tilde{c}_e) - RT\upsilon_w(c_e - \tilde{c}_e) \quad [7]$$

Using [6] and [7], Eqn [2] is transformed into

$$\Delta F = N_i[\mu_i(P_w,T) - \mu_w(P_w,T,\tilde{c}_e) + RT\upsilon_w(c_e - \tilde{c}_e)] + \sigma S_i \quad [8]$$

The equation $\mu_i(P_w,T) = \mu_w(P_w,T,\tilde{c}_e)$ determines the dependence of the melting point of a solution upon its initial concentration (5) and represents the melting diagram of the solution. In accordance with this, the value $\tilde{c}_e$ in the equation [8] can be interpreted as the concentration of an aqueous solution where the melting point of that solution is T. In view of the last equation, we can write [8] in the form

$$\Delta F = N_i RT\upsilon_W(c_e - \tilde{c}_e) + \sigma S_i \quad [9]$$

From Eqn [5] for a spherical ice nucleus, it follows that:

$$R_i = \left(\frac{3\upsilon_i}{4\pi}\right)^{\frac{1}{3}} N_i^{\frac{1}{3}} \quad \text{and} \quad S_i = 4\pi R_i^2 = (4\pi)^{\frac{1}{3}}(3\upsilon_i)^{\frac{2}{3}} N_i^{\frac{2}{3}}$$

According to this equation, Eqn [9] is transformed into

$$\Delta F = N_i RT\upsilon_w(c_e - \tilde{c}_e) + (4\pi)^{\frac{1}{3}}(3\upsilon_i)^{\frac{2}{3}}\sigma N_i^{\frac{2}{3}} \quad [10]$$

Thus, when $c_e < \tilde{c}_e$, the change in the free energy of the thermodynamic system during formation of a spherical ice crystal in a solution depends on the number of water molecules N_i contained in the crystal and is not monotonous. It has an extreme value when the number of water molecules in the crystal is equal to

$$N_i^* = \frac{32}{3}\frac{\pi\upsilon_i^2\sigma^3}{(RT)^3\upsilon_w^3(\tilde{c}_e - c_e)^3} \quad [11]$$

The value $\Delta F(N_i^*) = \frac{16\pi}{3}\frac{\upsilon_i^2\sigma^3}{(RT)^3\upsilon_w^2(\tilde{c}_e - c_e)^2}$ represents the activation energy for the process of ice formation in a supersaturated solution, and the average time required to form an ice crystal in a supercooled solution, according to the general formula [1] is

$$\langle t\rangle_i = C\exp\left[\frac{16\pi}{3kT}\frac{\upsilon_i^2\sigma^3}{(RT)^2\upsilon_w^2 c_0^2\left(\frac{c_e}{c_0} - \frac{\tilde{c}_e}{c_0}\right)^2}\right] \quad [12]$$

Obviously at an infinitely large supersaturation of a solution $\tilde{c}_e - c_e$, the time of ice crystal formation $\langle t\rangle_i$ must tend to zero. To satisfy this requirement, let $C \approx \frac{A}{x^2}$. Then

$$\langle t\rangle_i \approx \frac{A}{x^2}\exp\frac{B}{\left(\frac{T}{T_{k0}}\right)^3}x^2, \quad [13]$$

where $x \equiv \frac{\tilde{c}_e}{c_0} - \frac{c_e}{c_0}$ is T_{k0}- the value of the solution melting point with the initial concentration (before freezing) of the dissolved substance c_0,

$$B \equiv \frac{16\pi\upsilon_i^2\sigma^3}{3kT_{k0}(RT_{ko})^2\upsilon_w^2 c_0^2}$$

To satisfy instantaneous crystallization of water (<0.1 s) at its lowest possible supercooling temperature (–40°C) (4,7) we can assign 0.15 min to A at homogeneous ice formation. According to equation [12], the probability of intracellular ice formation per unit time in oversaturated intracellular solution is

$$W_1 = \frac{1}{\langle t\rangle_i} = \frac{\left[\bar{\tilde{c}}(\bar{T}) - \bar{c}_{in}\right]^2}{A} \times \exp\left\{-\frac{B}{\bar{T}^3\left[\bar{\tilde{c}}(\bar{T}) - \bar{c}_{in}\right]^2}\right\} \quad [14]$$

where $\bar{\tilde{c}} \equiv \frac{\tilde{c}}{c_0}$, $\tilde{c}(\bar{T})$ is the concentration of the extracellular solution, at which this solution is in a thermodynamic equilibrium with ice at a temperature T, $\bar{T} = \frac{T}{T_{k0}}$ is the normalized temperature; T is the current value of absolute temperature; $\bar{c}_{in} = \frac{c_{in}}{c_0}$ is the normalized concentration of intracellular solutes; c_{in} is the current total molar value of intracellular solutes.

Therefore, the probability of intracellular ice formation in an oversaturated intracellular solution during extracellular crystallization is determined by an integral over time from the

time t_{k0}, at which crystallization in the extracellular solution starts, to the moment t_E, when the temperature of the extracellular solution becomes eutectic:

$$W^* = \int_{t_{k0}}^{t_E} W_1 dt \qquad [15]$$

As to the prevention of intracellular ice crystal formation, an optimal cooling mode is $T(t)$, when the probability of intracellular crystallization is minimal in comparison to other modes, i.e. the mode at which

$$W^* = \int_{t_{k0}}^{t_E} \frac{\left[\bar{\bar{c}}\left(\bar{T}\right) - \bar{c}_{in}\right]^2}{A} \times$$

$$\times exp\left\{-\frac{B}{\bar{T}^3\left[\bar{\bar{c}}\left(\bar{T}\right) - \bar{c}_{in}\right]^2}\right\} dt \to min \qquad [16]$$

According to the two-factor theory, ice crystal formation in a cell unavoidably results in cell death. Therefore, the probability W^* can be equated with the probability of cell death due to intracellular crystallization.

However, the damage to cells during crystallization in a cell suspension is caused not only by formation of intracellular ice crystals but also by so-called solution effects. As a result, the damage increases with a higher concentration of solutes being in contact with cellular structures, and with the time of cell exposure to increased concentration of solutes (2,10,13). To account for the contribution of these effects to cell damage during crystallization, the following simple dependence of probability of cell damage due to the concentrations of extra- and intracellular hypertonic solutions and time of exposure can be applied

$$W^{**} = \frac{1}{2D} \int_{t_{k0}}^{t_E} \left(\bar{c}_{in} \bar{\bar{c}}\right) dt \qquad [17]$$

Biological interpretation of constant D in Eqn [17] is as follows: it equals the duration of exposure to a cryopreservation solution at the temperature of its melting, during which 50% of cells lose their viability. Choosing sub-integral expression in a form of a product of extra- and intracellular solutions concentrations takes into account of the fact that the damage of structural and functional cell elements can take place both as a result of direct contact of the external surface of cell membrane with an extracellular surrounding solution, and as a result of damage of sub-cellular structures with intracellular hypertonic solution. In the case of cell freezing the following condition holds $\bar{c}_{in} \neq \bar{c}_{out}$ (18).

It should be noted that yeast *Saccharomyces cerevisiae* show the relatively high resistance to moderate osmotic loading: exposure to 1.28 M DMSO in aqueous 0.15 M NaCl at –4°C for 12 hours reduces their colony-forming ability only by 50%. This corresponds to the value of D = 720 min (16). Murine enterocytes, by contrast, were sufficiently sensitive to the action of solutions (15). Our studies show that the vitality of these cells significantly decreased after 30 min of incubation in saline solutions of cryoprotectants. The least damaging was the solution of 1, 2-propanediol in a salt buffer. Therefore, for these cells we selected the value of the coefficient D = 15 min.

Both the solution effects and intracellular crystallization affect cells independently from each other. Therefore, the probability of cell damage W due to crystallization of a cellular suspension during freezing equals the sum of the above probabilities [16] and [17].

$W = W^* + W^{**}$. At a fixed constant cooling rate $\frac{dT}{dt} = -\beta\left(\beta \succ 0\right)$ we get

$$W = \frac{T_{k0}}{\beta A} \int_{t_{k0}}^{t_E} \left[\bar{\bar{c}}\left(\bar{T}\right) - \bar{c}_{in}\right]^2 exp\left\{-\frac{B}{\bar{T}^3\left[\bar{\bar{c}}\left(\bar{T}\right) - \bar{c}_{in}\right]^2}\right\} d\bar{T} -$$

$$-\frac{T_{k0}}{2D\beta} \int_{t_{k0}}^{t_E} \left(\bar{c}_{in} \bar{\bar{c}}\right) d\bar{T} = min \qquad [18]$$

As a frozen suspension contains a large number of cells, the value of W 100% determines the percentage of cells damaged in the process of crystallization of a cellular suspension.

In order to find the dependence of the percentage of damaged cells upon cooling rate based on the derived expression, we first have to define the dependences $\bar{\bar{c}}\left(\bar{T}\right)$ and $\bar{c}_{in}\left(\bar{T}\right)$ that appear in Eqn [18]. We used yeast (*S. cerevisiae*) suspension in 1.28M DMSO in physiological solution (16) and murine enterocytes suspension in 1M 1, 2-propanediol in physiological solution (15). The melting diagram in coordinates of the total mass of dissolved substances on the melting temperature can be obtained by a second-order polynomial approximation of the melting curve on the normalized temperature by the least-squares method (19). Thus, the required dependence for the estimation of the optimal constant cooling rate for *Saccharomyces cerevisiae* and murine

enterocytes has the following forms, respectively:

$$\bar{\tilde{c}}\left(\bar{T}\right) = -83,593\bar{T}^2 + 125,95\bar{T} - 41,362 \quad [19]$$

$$\bar{\tilde{c}}\left(\bar{T}\right) = -127,53\bar{T}^2 + 169,91\bar{T} - 41,769 \quad [20]$$

The dependence $\bar{c}_{in} = \bar{c}_{in}\left(\bar{T}\right)$ is described in (3), and with regard to the case examined here can be presented as

$$\frac{dy}{dt} = \frac{1}{\tau_w}\left[\frac{1-\alpha}{y-\alpha} - \frac{\tilde{c}\left(T\right)}{c_{k0}}\right] \quad [21]$$

$$c_{in} = c_{k0}\frac{1-\alpha}{y-\alpha} \quad [22]$$

where $y = \frac{V}{V_0}$ is the relative cell volume; V and V_0 are the current and initial value of cell volume respectively; t is time; α is the partial volume of osmotically inactive intracellular substances; c_{in} is the molar concentration of the substances in the cell; c_0 is the initial concentration of the intracellular solution, before crystallization, $\tau_w = \frac{1}{\gamma L_p \pi_0}$ is the typical time taken for water molecules to penetrate through a cell membrane; γ is the ratio of the cell surface area to its initial volume; L_P is the filtration coefficient of the cell membrane, π_0 is the initial (at the melting temperature) osmotic pressure of extracellular solution.

Combining Eqn [21] and [22], we get

$$\frac{d}{dt}\bar{c}_{in} = -\frac{\bar{c}_{in}^2}{(1-\alpha)\tau_w}\left[\bar{c}_{in} - \bar{\tilde{c}}\left(\bar{T}\right)\right] \quad [23]$$

Taking into account of the Arrhenius' law

$$\tau_w\left(T\right) = \tau_w\left(T_{k0}\right)exp\left[\frac{E}{RT_{k0}}\left(\frac{1}{\bar{T}} - 1\right)\right] \quad [24]$$

where E is the activation energy of the transmembrane transfer of water molecules; R_0 is the universal gas constant; the definition $\frac{dT}{dt} = -\beta$, instead of [23] we get

$$\frac{d\bar{c}_{in}}{d\bar{T}} = \frac{T_{k0}\bar{c}_{in}^2 exp\left[\frac{E}{RT_{k0}}\left(1 - \frac{1}{\bar{T}}\right)\right]}{(1-\alpha)\tau_w\left(T_{k0}\right)\beta}\left[\bar{\tilde{c}}\left(\bar{T}\right) - \bar{c}_{in}\right] \quad [25]$$

RESULTS AND DISCUSSION

Solving Eqn [25] numerically at different cooling rates β and initial conditions $\bar{c}_{in}\left(0\right) = 1$,

Table 1. Cellular Characteristics Used in the Calculations

Cell type	L_P, $m^3/N{\cdot}s$ Filtration coefficient	E, kJ/mol Water transfer activation energy	γ, μ^{-1} Cell surface-volume ratio	α Osmotically inactive volume	D, min Constant in expression [17]
Saccharomyces cerevisiae (17)	0.54×10^{-14}	24.5	1.32	0.27	720
Murine enterocyte (14,15)	0.7×10^{-14}	38	0.53	0.51	15

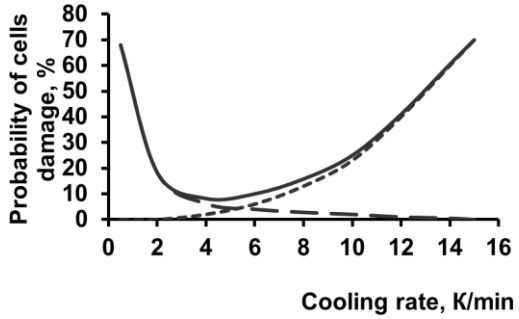

Figure 1. Probability of cell damage in yeast suspension due to the total contribution of solution effects (– – –) and intracellular crystallization (- - - -), depending on the cooling rate during freezing in 1.28 M DMSO and 0.15M NaCl.

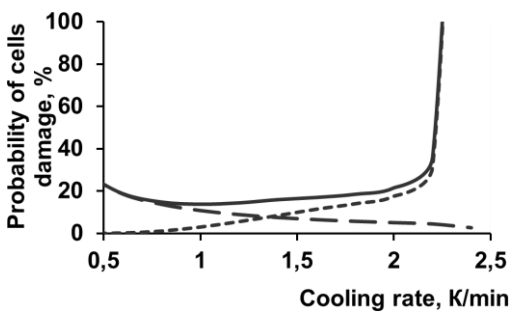

Figure 2. Probability of murine enterocytes cell damage due to the total contribution of solution effects (– – –) and intracellular crystallization (- - - -), depending on the cooling rate during freezing in 1M 1,2-propanediol and 0.15M NaCl.

$\bar{T}(0)=1(\bar{T}_E \le \bar{T} \le 1)$ and taking into account of the characteristics of yeast cells (*S. cerevisiae*) (17) or murine enterocytes (14,15), such as L_P - cell membrane filtration coefficient, E - the activation energy of the transmembrane transfer of water molecules, α - the partial volume of osmotically inactive intracellular substances, γ - the ratio of cell surface area to its initial volume (Table 1), T_{k0}=268,35K for 1.28M dimethyl sulfoxide and T_{k0}=270,4K for 1 M 1,2-propanediol (19), σ =0.03 Н/м (4), as well as Eqn [19] and [20] that determine $\bar{\varepsilon}(\bar{T})$, we find the probability of cell damage during crystallization at constant cooling rates β (Figure 1,2) by substitution of the solution in Eqn [18].

As follows from the results of the calculation presented in Figures 1 and 2, the cryodamage in the range of cooling rates $\beta \le 3$ K/min for yeast cells and β≤1K/min for murine enterocytes takes place only due to the effects of solution, which are determined by an integral [4]. While at cooling rates $\beta \ge 4$ K/min for yeast cells and $\beta \ge 2.5$ K/min for murine enterocytes, the cryodamage is mainly a result of intracellular crystallization, the contribution of which is determined by the integral [3].

Thus, we consider a constant cooling rate of 1–1.5 K/min and 5 K/min to be optimal for freezing suspensions of murine enterocytes under protection of 1 M 1,2-propanediol and of yeast cells under protection of 1.28 M (10%) dimethyl sulfoxide, respectively. Despite the assumptions that we made to calculate the probability of cell damage during freezing, our theoretical estimation agrees very well with experimental results (1,6,15,16). The algorithms that we used for theoretical estimation of the optimal cooling rates in linear freezing modes proved to be applicable for suspensions of cells, which differ significantly in their sensitivities to different types of damaging factors. Therefore, the proposed algorithm is suitable for the development of cryopreservation methods for different cell suspensions.

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