Title:

Cooling-rate dependence of the cryopreservation of aquaporin-overexpressing cells with a non-permeable cryoprotectant.

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Abstract

Dehydration of intracellular water is an important factor in the cryopreservation of cells, but questions remain as to the appropriate amount and timing of dehydration and the detailed mechanism of the freezing process. Answering these questions will lead to improvements in cryopreservation methods that have remained unchanged for more than half a century and to an increase in the number of cell types that can be cryopreserved. Therefore, we aimed to reveal the time point when cells were dehydrated in their cooling process and how much their viabilities were improved by dehydration. We conducted cryopreservation experiments using cells with enhanced water permeability due to membrane overexpression of the water transport channel protein (AQP4). The AQP4-expressing cells or non-AQP4-expressing cells were cryopreserved under different cooling rates after addition of the membrane-permeable cryoprotectant (CPA) Me_2SO, the non-membrane-permeable CPA trehalose, or no CPA. The results showed that no cryopreservation was successful without CPAs, even in the AQP4-expressing cells with increased water permeability. At slow freezing rates below 35°C/min, viability with Me_2SO was maintained with decreasing in the cooling rate, but with trehalose, the viability decreased. At cooling rates above 80°C/min, the viability of AQP4-expressing cells was significantly higher than that of AQP4-non-expressing cells. These results suggest that dehydration due to the osmotic-pressure difference generated after extracellular freezing is fatal to cells.

1. Introduction

Although cryopreservation (freeze–thawing) is a useful method of long-term cell preservation, the viability of the freeze–thawed cells is decreased after freeze–thawing. This effect is thought to be related to the formation of ice crystals within the cells. Ice crystal formation inside the cells also causes damage to the cell membrane, but if the solute concentrations inside the cell are sufficiently high due to dehydration, it would be expected that ice crystal formation inside the cell would be inhibited [14]. Viability after cryopreservation is also known to depend on the cooling-rate [3][14][15]. In slow freezing, extracellular water freezes first, creating a concentration gradient of solutes inside and outside the cell. Therefore, the intracellular concentration increases as water flows from the inside to the outside of the cell, and intracellular freezing is inhibited. However, the highly concentrated solution formed by the extracellular ice-crystal formation may damage the cells. On the other hand, in rapid freezing, supercooling and intracellular ice-crystal formation (IIF) occur within the cell during cooling, resulting in cellular damage that is thought to reduce survival after freezing [14]. This binary mechanism of freezing injury corresponds to Mazur's "two-factor hypothesis" [15], which states that intracellular ice formation and intracellular solute accretion are responsible for the cellular damages under conditions of rapid and slow cooling, respectively. Nonetheless, while it is true that the dehydration of intracellular water is an important factor in cryopreservation, the timing and appropriate amount of dehydration are not clear, and therefore the freezing process is not fully understood.

Cryoprotectant agents (CPAs) are used in both slow freezing and rapid freezing methods to increase viability. Dimethyl sulfoxide (Me_2SO) is the main CPA used when preserving cells for storage, but because Me_2SO is toxic to cells [12][20], its clinical application can result in severe side effects. As an alternative, disaccharides—which are used by plants and animals to acquire freezing tolerance—have been investigated as potential CPAs [2][4][6][16]. For example, instead of using cell membrane-permeable Me_2SO as a CPA to inhibit ice crystal formation inside and outside the cell, the disaccharide trehalose, which is not permeable to the cell membrane due to its molecular size, has been selectively transported into cells via a trehalose transporter and shown to successfully improve viability [18][19]. When normal cells are transferred from normal medium to a medium supplemented with trehalose, an osmotic-pressure difference is created between the inside and outside of the cells. This pressure difference dehydrates the cells in the trehalose-containing medium.

It was shown in the first section that inhibition of intracellular ice crystal formation is important for improving the viability of cryopreserved cells, and that slow freezing is thought to dehydrate the cell interior, thereby preventing damage to the cell membrane during the freezing process. Therefore, in this study we focused on the effects of increased rate of dehydration by using cells that overexpress aquaporins. Aquaporins (AQPs) are a family of water channel proteins that are expressed in the plasma membrane and increase its water permeability [17]. There are 13 known AQPs, ranging from AQP0 to AQP12[1][5]. In this study we used cells overexpressing AQP4 [7], which is mainly expressed in the human brain; these cells were useful for our experiments because

they were also used in a previous study [10] on cryopreservation experiments. In that report, under a fast freeze rate condition of -120°C/min, the AQP4M1-overexpressing cells showed high viability that was almost identical to that under slow freeze rate conditions, while the AQP4 non-expressing cells were barely preserved. This indicated that AQP4-expressing cells have freeze-tolerance at a high cooling rate. As to the explanation for this phenomenon, one hypothesis is that the difference in water-transport properties between transport through aquaporins alone and transport through lipid bilayers alone is responsible for the freeze-tolerance of AQP4-expressing cells. Water diffusion across the lipid bilayer is temperature-dependent and water permeability decreases at low temperatures, but AQP4 remains permeable even at low temperatures, so AQP4-expressing cells are expected to dehydrate sufficiently to maintain high viability even at high cooling rates [10].

In this study, we investigated the time point when cells were dehydrated in their cooling process and the degree to which their viabilities were improved by dehydration. To accelerate dehydration, we added trehalose, which also acts as a non-membrane-permeable CPA, to the solution. The cells began to be dehydrated by the osmotic-pressure difference immediately after their culture solution was replaced with a trehalose solution before freezing. To determine whether increasing the dehydration rate improves the viability of the cryopreserved cells, we used AQP4-overexpressing cells, which have cell membranes with high water permeability. The temperature or time starting for the extracellular freezing was varied by changing the cooling-rate. When the extracellular freezing occurs, cells in the non-frozen area are concentrated in the freeze-concentrated

phase, and therefore the cells would be dehydrated again after the extracellular ice-crystal formation. By comparing the results of these experiments using trehalose or Me_2SO_4 as the membrane permeable CPA, we were able to investigate how the freeze–thaw viabilities of AQP4-overexpressing cells and normal cells depend both on the cooling-rate and the CPA used.

2. Materials and Methods

2.1. Cell culture

Stable Chinese hamster ovary cells (CHO cells) overexpressing AQP4M1 (CHO-M1) and AQP4M23 (CHO-M23) and CHO-vector cells were established as in a previous study [7]. AQP4 is a member of the AQP family of water channel proteins. AQP4M1 and AQP4M23 are isoforms of AQP4 that initiate translation at methionine residues M1 and M23, respectively [13]. Although their differences in their arrangement, these two isoforms have similar water permeabilities and no known functional differences [5]. The cells were cultured in Nutrient Mixture F-12 HAM (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA), 1% penicillin/ streptomycin (Thermo Fisher Scientific), 1% G418 sulfate solution (50 mg/mL animal-derived-free) (Nacalai Tesque, Kyoto). Cell cultures were added to 75 cm^2 flasks (As One, Osaka, Japan) and incubated in a CO_2 incubator (MCO-18AIC; Sanyo, Osaka, Japan) at 37°C under 100% humidity and 5% CO_2-95% air.

2.2. Preparation of cell suspension

When the cells reached more than 80% confluence, they were dissociated by trypsin (0.05% trypsin-EDTA solution; Thermo Fisher Scientific). Then, 1 ml of the cell suspension (about 10^5 cells/ml) was put into a 1.8 ml cryotube vial (Thermo Fischer Scientific). The cells were collected by centrifugation at 1000 rpm for 3 min, and then the cell culture medium was replaced with 1 ml of freezing solution, which consisted of the culture medium supplemented with 500 mM trehalose. This trehalose concentration was selected as the optimal concentration at which the cryoprotective effect was maximal in preliminary experiments on the same cell lines to check the trehalose-concentration dependence of the freeze-thaw viability at a cooling rate of about 10°C/min.

2.3. Cryopreservation protocol

The cryopreservation protocol was identical to that used in a study by Gómez-Fernández et al. [6]. To control the cooling rate, we used various temperature conditions for freezing. In brief, cell-suspended samples were frozen in deep freezers set at –38°C (SC-DF25; Twinbird, Niigata, Japan), −60°C (VT-78; Nihon Freezer, Tokyo) and −80°C (VT-78). Another sample was placed in a Bicell freezer box (Japan Freezer, Tokyo) and frozen in a −80°C deep-freezer, which provides a cooling rate of 2-4°C/min. The following method was used to set faster cooling rates. First, the samples were set in the vapor cloud above liquid nitrogen or immersed in liquid nitrogen. Then, after 1–2 h, they were placed in a −80°C deep-freezer and stored for 1 week. The respective cooling rates were calculated

as the maximum temperature gradient after the start of freezing [6].

To investigate the cryoprotective effect of trehalose as a non-membrane permeable CPA, the same experiment was performed using Me_2SO 10% (v/v) as a membrane-permeable CPA, or medium with no CPA as a negative control.

2.4. Viability and growth assays

After one week of storage, the samples were thawed in a water bath at 37°C for about 5 min until the ice in the tubes had thawed completely. The samples were rinsed twice with 1 ml of medium by centrifugation and the supernatant was removed.

Viability was assessed using a flow cytometer (MUSE™; Cytek Biosciences Japan, Tokyo). 30 μL of sample and 270 μL of Muse™ reagent from a Muse Count & Viability Assay Kit (Cytek) were added to a 1.5 mL microtube (As One) and left at room temperature for at least 5 min to stain.

The remaining cell suspension was plated in a 12-well (As One) or 24-well culture plate (As One) to check the adhesion and proliferative functions for the live cells by microscopic observation. The cells were incubated in a CO_2 incubator and images were captured using a phase-contrast microscope (IX-71; Olympus, Tokyo) through a ×10 objective lens (UPlanFL10×PH; Olympus) and collected with a charge-coupled device (DP70; Olympus) every day after thawing for at least 1 week.

2.5. Observation of cell dehydration

In order to quantify the amount of dehydration during the experiments, we used a phase-contrast microscope (IX-71) to observe the volume change of the cells in the CPA-containing media. 1 ml of the cell suspension (about 10^5 cells/ml) was put into a 1.8 ml cryotube vial (Thermo Fisher Scientific). Samples were replaced with 1 mL of culture medium containing 500 mM trehalose by centrifugation and pipetted. The samples were seeded into 5 cm-diameter petri dishes containing 1 ml of 500 mM trehalose medium. Samples in Me_2SO 10% medium were also prepared in the same way. The petri dish was placed on a stage of the microscope and maintained at 37°C for 5 min. After checking that the floating cells had settled and could be clearly observed, we captured 10–15 images using the phase-contrast microscope through a ×20 objective lens (LUCPlanFLN20×PH). This observation was carried out for 5 to 120 min and images were taken every 30 min. The samples were kept still in the incubator except for the time when the images were taken. In order to perform these same observations at low temperatures, we used the following setup on the microscope stage. A low-temperature bath circulator (AC150; Thermo Fisher Scientific) was used to supply temperature-controlled antifreeze solution to a box-shaped thermostatic chamber containing the sample to maintain a sample temperature of 4 ± 1°C. The temperature was measured by a thermocouple set inside the chamber. Samples were left on the stage in the thermostatic chamber except during observation.

The images were analyzed using ImageJ (National Institutes of Health, Bethesda, MD). Cells were selected by setting a threshold value for each image, and the area of the cell was

measured using the “Analyze Particles” function, and then each cellular radius was calculated from the measured area using Igor Pro 6 (HULINKS, Tokyo). Histograms were generated from the obtained radii, and then the radii underwent lognormal fitting by Igor Pro 6. The peak value of the lognormal fit was taken as the average value of the radius and the uncertainty of the average radius was taken as the standard deviation of the fitting. The volume was determined from the average radius when approximating the cell as a sphere. The volume was calculated at each time point and the cell volume was standardized by dividing it by the initial volume of the cell obtained by averaging the radii in the first 5 min.

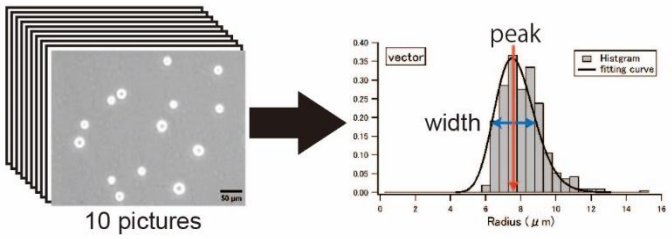


Fig. 1. Histograms of cell radii and fitting examples. We took 10 pictures of each sample and measured their radii.

2.6. Apoptosis assay

In the two-factor hypothesis [15], the cells are damaged by the high-osmotic pressure in the hypertonic solution. However, the precise time point at which this “solution effect” inflicts its damages remains unclear. To clarify this, we investigated the health of cells exposed to different freezing solutions before freezing, by measuring the level of apoptosis [11] as follows. A 1-ml

184 portion of the cell suspension was removed and replaced with 1 ml of 500 mM trehalose at 37°C, and
185 then the solution was placed in a refrigerator maintained at 4°C for 0 to 120 min. Subsequently, the
186 samples were rinsed twice with 1 mL of medium. A 100 μL aliquot of sample was added to a
187 microtube containing 100 μL of Muse™ reagent from an Annexin V & Dead Cell Kit (Cytek), and
188 then left at room temperature for at least 20 min for staining. The level of apoptosis in the sample
189 was then measured using a Muse™ Cell Analyzer in Annexin V & Dead Cell mode.
190
191 3. Results and Discussion
192 3.1. Cooling-rate dependence of the freeze-thaw viability
193 3.1.1. Cooling-rate dependence of the viability of cells frozen with Me_2SO
194 To confirm the reliability of the research methods in this study, we first measured the cooling-
195 rate dependence of the freeze-thaw viability of cells frozen with 10% Me_2SO (Fig. 2), which was
196 used in a previous study [10].
197 In the absence of Me_2SO, viability was lower than approximately 10% regardless of the
198 cooling rate (Fig. 2; No CPA). This indicates that the cryopreservation of CHO cells without CPAs
199 was not successful in this cooling rate range, even if AQP4 was overexpressed on their cell
200 membranes. Since the two-factor hypothesis suggests that promoting dehydration during cell
201 freezing and inhibiting IIF improves viability after thawing, we had expected that the cells with
202 improved water permeability in the membrane could be cryopreserved without CPAs. However, our

finding of low viability irrespective of cooling rate indicates that simply increasing the membrane water permeability is not sufficient, and the cells require the protective effects conferred by CPAs to remain viable.

Under 10% Me_2SO conditions, all CHO cells overexpressing AQP4M1 (CHO-M1) or AQP4M23 (CHO-M23), and all vector cells (CHO-vector) showed similar viability trends at cooling rates less than 100°C/min (Fig. 2; Me_2SO). An especially high viability of around 90% was observed at cooling rates less than 40°C/min. At cooling rates from 40°C/min to 100°C/min, the viability decreased as the cooling rate increased for all cell lines.

At cooling rates above 100°C/min, on the other hand, the viability of CHO-vector cells was as small as that without CPA (Fig. 2; Me_2SO); that is, the cells were not cryopreserved under these conditions. However, both CHO-M1 and CHO-M23 cells showed viabilities of around 20%, which was clearly higher than that of CHO-vector cells. Since these results were quantitatively consistent with the findings in the previous study [10], the reliability of the experimental method in this study was confirmed. We also found that CHO-M23 cells, which had not been reported in the previous study [9], had a similar cooling-rate dependence on viability as that of CHO-M1 cells, and also had a freeze-tolerance similar to that of CHO-M1 cells under the high cooling rate conditions.

We cultured cells after thawing to check their adhesion and proliferative functions after cryopreservation. Figure 3 shows micrographs of CHO-M1, CHO-M23 and CHO-vector cells cryopreserved at cooling rates above 100°C/min and then thawed and cultured for 5-6 days. Both

CHO-M1 and CHO-M23 cells proliferated well, while the CHO-vector cells did not. This confirmed that the surviving cells whose viabilities were confirmed by flow cytometer maintained their adhesion and proliferative functions. Thus, we used the viability measured by flow cytometry as the index of the health of cryopreserved cells in this study.

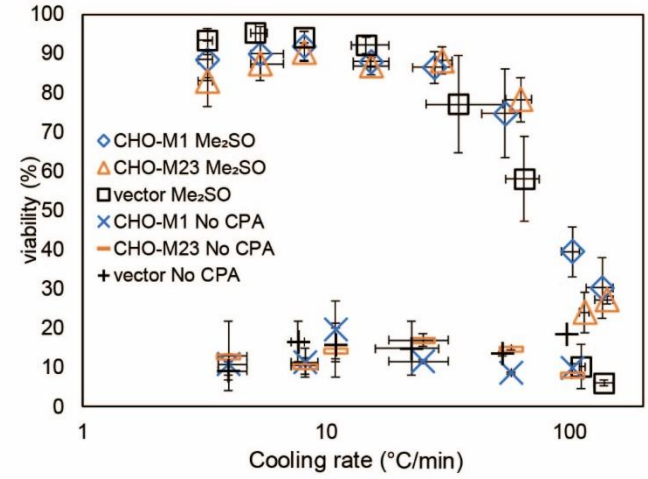


Fig. 2. Viability assessed using a flow cytometer (MUSE^{TM}; Cytek Biosciences Japan, Tokyo) after freeze-thawing in 0% and 10% Me_2SO solutions. CHO-M1 indicates CHO cells overexpressing AQP4M1, CHO-M23 indicates CHO cells overexpressing AQP4M1 and vector means CHO-vector cells.

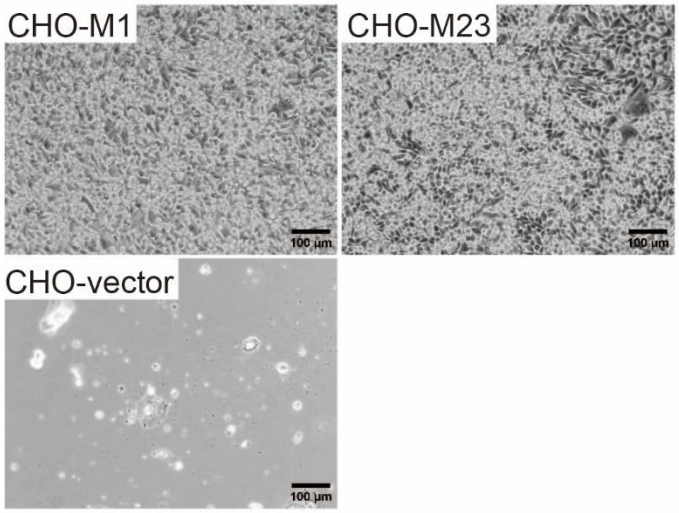


Fig. 3. Observation of cultures after freeze–thawing of 10% Me_2SO under rapid-freezing conditions. CHO-M1 (top left) were frozen at 105°C/min, stored for 1 week at –80°C, viability was 42.3%, and then cultured for 5 days. CHO-M23 (top right) were frozen at 120°C/min, stored for 1 week at –80°C, viability was 18.8%, thawed, and then cultured for 6 days. CHO-vector (bottom left) were frozen at 126°C/min, stored for 1 week at –80°C, determined by flow cytometry to have a viability of 6.9%, thawed, and then cultured for 6 days.

3.1.2. Cooling-rate dependence of the viability of cells with a non-membrane-permeable CPA

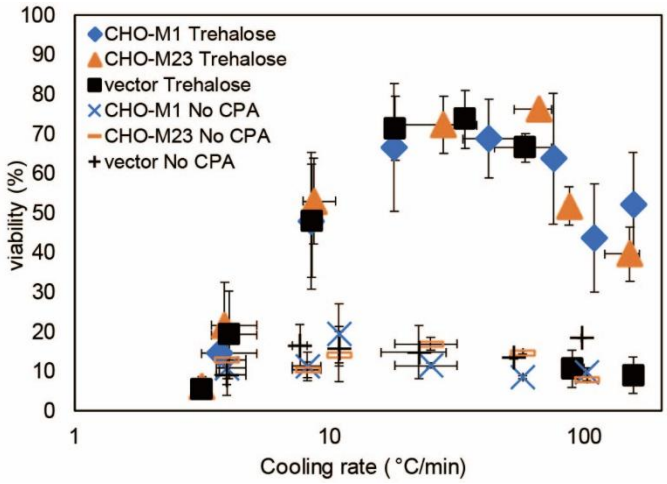
We then investigated the cooling-rate dependence of the viability of cells in a solution of 500 mM trehalose, which is a non-membrane-permeable CPA (Fig. 4). Since trehalose cannot penetrate cells spontaneously, its cryoprotective effect on CHO-K1 cells is considered to be relatively low (Uchida et al., 2019 [19]; see the section “Cooling-rate dependence of CHO-TRET1 vector cells”). However, as shown in Fig. 4, while high viability was obtained even in CHO-vector cells under certain freezing conditions with trehalose, no cryopreserved cells were observed in the absence of

247 trehalose. Therefore, we consider that trehalose plays a cryoprotective role even under the
248 extracellular condition.

249 The cooling-rate dependence of the viability of cells frozen with 500 mM trehalose showed a
250 bell-shaped curve, with a maximum viability of about 70% at about 35°C/min for all three cell lines
251 (Fig. 4; Trehalose). At cooling rates below 35°C/min, the viability decreased as the cooling rate
252 decreased, and at cooling rates below 5°C/min, the viability was similar to that without CPA. In
253 addition, at cooling rates below 80°C/min, there was no significant difference between CHO-M1 or
254 CHO-M23 cells and CHO-vector cells. This indicates that AQP4-overexpression had no effect on
255 viability under these lower cooling rate conditions.

256 On the other hand, at cooling rates above 80°C/min, the viability of CHO-vector cells was
257 suddenly decreased to that of the cells without CPA (Fig. 4; Trehalose). However, CHO-M1 and M23
258 cells retained higher viabilities of about 50%, even though their viabilities decreased slightly with
259 increases in the cooling rate. Therefore, we confirmed that CHO-M1 and M23 cells have similar
260 levels of freezing tolerance at cooling rates higher than 80°C/min when trehalose is used as CPA,
261 even though it does not penetrate cells.

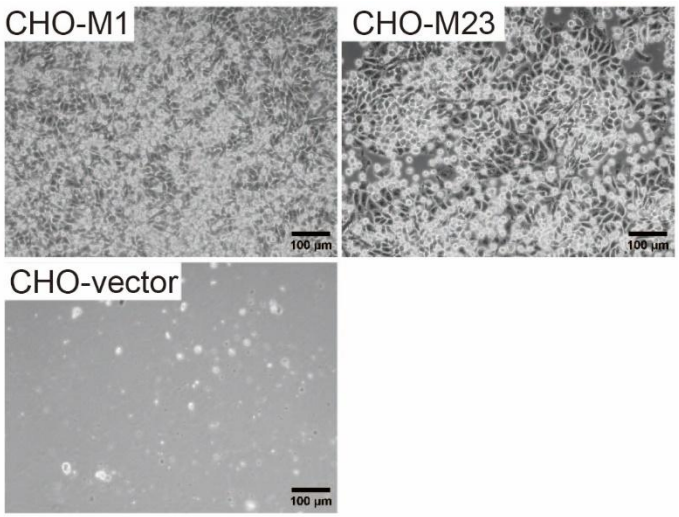
262 We next cultured cells to check their health as in the previous section (Fig. 5). We obtained
263 results similar to those under the Me_2SO condition and confirmed the accuracy of our viability
264 assessment.



266

267 Fig. 4. Cooling-rate dependence of the freeze-thaw viabilities of CHO-M1, M23 and vector cells

268 with 500 mM trehalose (solid marks) and without trehalose (open marks).



269

270 Fig. 5. Observation of cultures after freeze–thawing of 500 mM trehalose under quick-freezing

271 conditions. All CHO-M1 (top left), CHO-M23 (top right) and CHO-vector (bottom left) cells were

272 frozen at 146°C/min, stored for 1 week at -80°C, thawed, and then cultured for 5 days; images show

273 the cells on day 5 of culture. The viabilities of CHO-M1, CHO-M23 and CHO-vector cells were

274 36.7%, 40.2%, and 6.1%, respectively.

275

3.1.3. Comparison of the cooling-rate dependence of viability between two types of CPA

Comparing the cooling-rate dependences between the two CPAs (Fig2; Me_2SO, Fig. 4; Trehalose), at cooling rates slower than 35°C/min, the viability was changed depending on the CPAs, but no significant difference between AQP4-overexpressing cells and vector cells. The viability decreased with decreasing cooling rate with trehalose, whereas with Me_2SO, the viability was maintained with decreasing in the cooling rate. Therefore, we consider that dehydration through AQP4 had no important effect on viability at cooling rate below 80°C/min. The viability decreases with decreasing in cooling rates with trehalose may be resulted from the 'solution effect' in the two-factor hypothesis, but shown in 3.3.1, further analysis revealed when they were suffered.

Although the viabilities at cooling rates from 35°C/min to 80°C/min were nearly the highest observed in this study, there was no difference in viability between CHO-M1 or CHO-M23 and CHO-vector cells regardless of the CPA used, and the viabilities of all cell lines tended to gradually decrease with increasing cooling rate. Therefore, the viabilities were controlled by dehydration through the cell membrane. The viabilities remained high, but they were still diminished in proportion to the increase in cooling rate. Such a decrease in viability with increasing cooling rate is consistent with the model using IIF in the two-factor hypothesis.

At cooling rates above 80°C/min, CHO-M1 and CHO-M23 cells showed higher viabilities than CHO-vector cells with both Me_2SO and trehalose. We confirmed that AQP4-overexpressing cells have freeze-tolerance at high cooling rates regardless of the presence of intra- and extracellular

CPAs. This condition will be discussed in Section 3.2. The result that viability is improved due to rapid dehydration by AQP is consistent with the two-factor hypothesis that dehydration suppresses IIF.

Despite the concordance between these results and the two-factor hypothesis model, it remains unclear when dehydration occurs to control survival rates, or to what extent dehydration must occur. To elucidate the timing of the critical dehydration, we measured the temperature profile of a solution sample. Figure 6 shows the temperature change when 1 ml of a medium containing 500 mM trehalose was frozen at 3.4°C/min. The duration from the start of cooling to the start of the ice formation (t_{f1}) was about 20 min, and between the start of the ice formation (t_{f1}) and its completion (t_{f2}) at this cooling rate was about 30 min. The time between t_{f1} and t_{f2} at the fastest cooling rate was about 5 seconds (Fig. S1 in Supplementary information). In order to separate the dehydration before (before t_{f1}) and after (between t_{f1} and t_{f2}) the freezing of the solution, we next investigated the apoptosis ratio and volume change of the cells due to the dehydration and the solution effect caused by CPA.

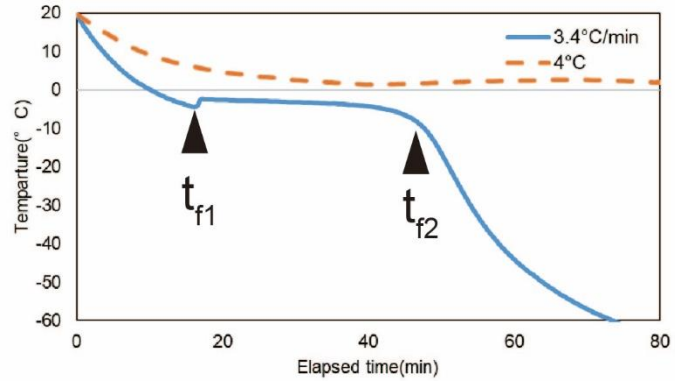


Fig. 6. Temperature change of 1 mL freezing solution containing 500 mM trehalose cooled at a

311 cooling rate of 3.4°C/min (solid line) and stored at 4°C (dotted line). t_{f1} and t_{f2} are the times of ice
312 formation and completion of freezing.
313
314 3.2. Evaluation of cell dehydration and apoptosis in CPA-containing culture medium
315 3.2.1. Apoptosis in CPA-containing culture medium
316 First, we investigated whether cells were damaged by immersion in the CPA-containing
317 culture medium before freezing. We measured the cell apoptosis ratio across the period of immersion
318 in the freezing solutions containing 500 mM trehalose at 4°C, since the temperature change of this
319 apoptosis experiment was very similar to that observed in the cooling process at 3.4°C/min (Fig. 6).
320 Figure 7 plots the apoptosis ratios of the three lines of cells against the immersion time up to 120
321 min. No increase in apoptosis ratio was observed in any of the cell lines until 60 min after
322 immersion. However, at 120 min, the CHO-vector and CHO-M23 cells incurred damages that
323 increased the apoptosis ratio significantly. These results indicated that that there was little or no cell
324 damage before the start of ice formation, and therefore the cell damage occurring in the freezing
325 solution containing trehalose would have taken place after the ice formation in the extracellular
326 solution.

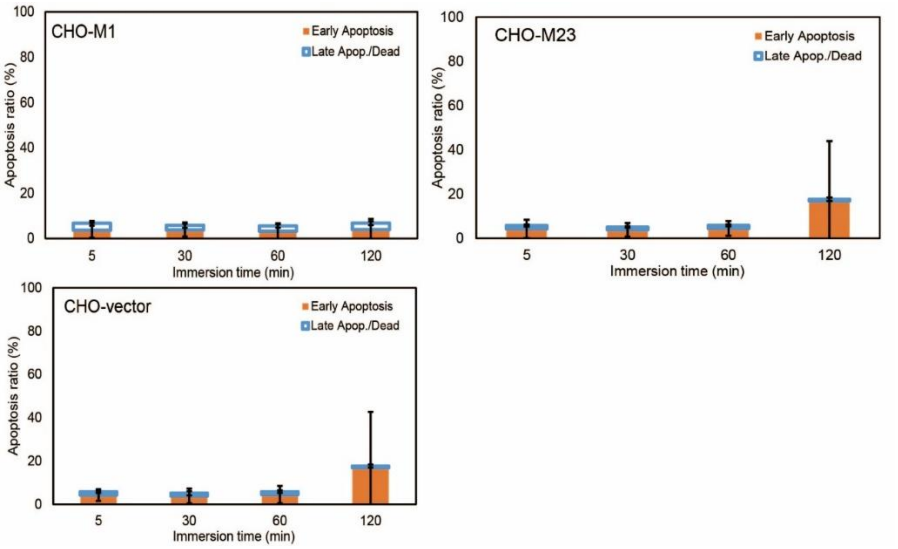


Fig. 7. Apoptosis ratio after immersion in a freezing solution containing 500 mM trehalose at 4°C for 5 to 120 min for CHO-M1, CHO-M23, and CHO-vector cells.

3.2.2. Cell volume changes by CPA

Next, we estimated the degree of dehydration in a CPA-containing culture medium before the start of the ice formation (t_{fI}). For each of the cell lines, phase-contrast microscopic images were used to measure the cell-volume change in a freezing solution containing either 500 mM trehalose or 10% Me_2SO at 37°C or 4°C, and then the volume change was used to estimate the extent of dehydration before cooling. The average volume ratio of the cell at time t after immersion was calculated as $V(t)/V_0$, where V_0 is the average initial volume. The volume change after immersion in the freezing solutions showed that all cell lines shrank significantly in the first 5 min and then retained almost constant size over the next 120 min (Fig. S2). We therefore compared the volume ratio of each cell line at 5 min under each condition.

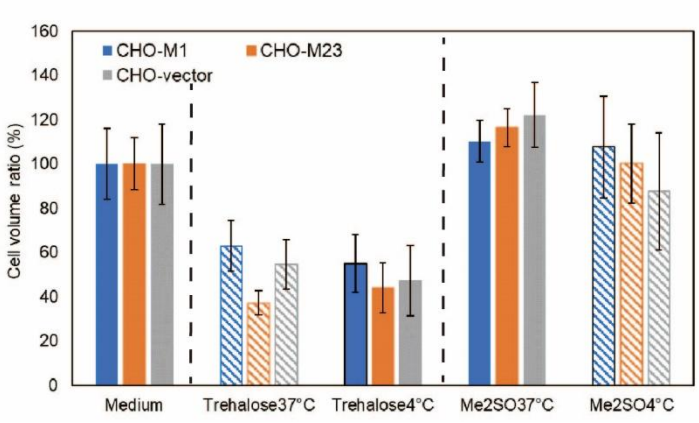


Fig. 8. Cell volume ratio after 5-min immersion.

Figure 8 shows that the values of V (5 min)/V_0 of all three cell lines were around 100% in a freezing solution containing 10% Me_2SO, indicating that little or no cell shrinkage occurred in the solution. On the other hand, the values of V (5 min)/V_0 of all three cell lines decreased to about 50% in a freezing solution containing 500 mM trehalose. This shrinkage trend was qualitatively similar under both the 37°C and 4°C conditions. A previous study [21] found that membrane permeability decreased at low temperatures for 5 min, so we considered that 5 min was sufficient to observe a water permeability difference. Under our present cryopreservation experimental conditions, the amount of dehydration at 4°C was found to be equivalent to that at 37°C after 5 min. These results show that CHO-M1, CHO-M23 and CHO-vector cells were dehydrated in the freezing solution containing trehalose soon after the exchange of their solutions. This dehydration may have indicated that both the osmotic dehydration through aquaporins and that through the cell membrane were completed within 5 min.

Our experimental protocol required more than 5 min from the exchange of the freezing

357 solutions to the start time of cooling. Therefore, dehydration due to osmotic pressure was completed
358 by the start time of cooling. Cell sizes before freezing did not differ among the three cell lines but
359 were significantly affected by the addition of CPA. Also, each cell was in an isotonic state with the
360 freezing solution. That is, the volume ratio in the Me_2SO-containing solution was about 100% at the
361 start of cooling, whereas that in the trehalose-containing solution was about 50%, which cannot
362 explain the viability difference. Therefore, the viability difference between CHO-M1 or CHO-M23
363 and CHO-vector cells under the rapid cooling condition would have resulted from the additional
364 dehydration that occurred during the subsequent cooling process (between t_{f1} and t_{f2}) as a result of
365 the extracellular ice crystal formation.
366 From Sections 3.2.1 and 3.2.2, we know that the dehydration process using CPA is complete
367 by the time cooling begins, and the apoptosis assay showed that the cells remain healthy for the time
368 period until freezing begins, i.e., before the ice formation (tf1). Therefore, it is thought that the cell
369 damage and dehydration between the start of ice formation (t_{f1}) and its completion (t_{f2}).
370
371 3.3. Effect of dehydration on freeze-thaw viability
372 We investigated the dependence of the viability of AQP4-overexpressing cells and non-
373 overexpressing cells on the cooling rate after freezing and thawing using Me_2SO, a permeable CPA,
374 and trehalose, a non-permeable CPA. The results were qualitatively consistent with the two-factor
375 hypothesis. To discuss the effect of IIF and the solution effect in greater detail, in the following

376 sections we will consider the viability variations in each range of freezing rates.

377

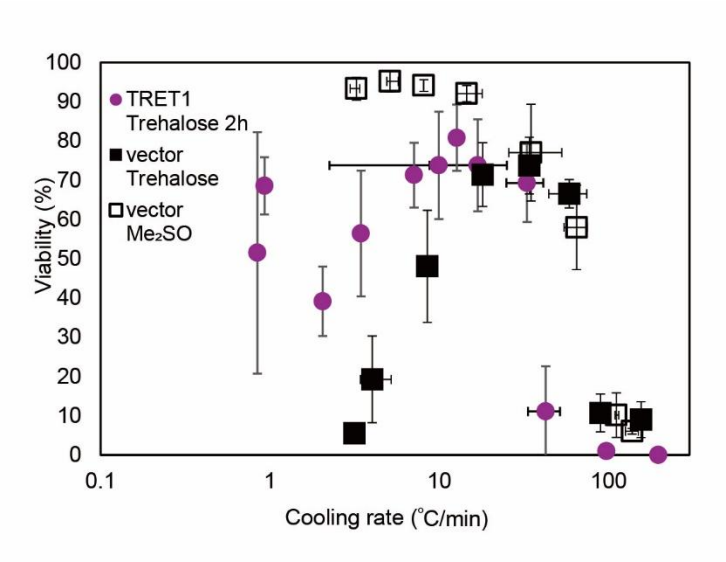
378 3.3.1. Effect of slow cooling on the freeze–thaw viability (below 35°C/min)

379 The viabilities under the slower cooling rate conditions differed according to the membrane-
380 permeable and -impermeable nature of the CPAs (Fig. 2; Me_2SO, Fig. 4; Trehalose). The viability of
381 each cell line in the freezing solution containing 500 mM trehalose decreased with decreasing
382 cooling rate (Fig. 4; Trehalose), whereas that in the freezing solution containing 10% Me_2SO
383 remained constantly high (Fig. 2; Me_2SO).

384 As discussed in Section 3.2, in this experiment, dehydration by CPA was complete in both
385 AQP4-overexpressing and non-overexpressing cells by the time the cells were dispersed in the
386 freezing solution and cooled. In addition, the extracellular freezing began (t_{f1}) within 20 min, and the
387 cells were not damaged during this time period. Because of the freeze-concentration phenomenon,
388 the cells were immersed in a higher concentration of solute between t_{f1} and t_{f2}. If the cells were in a
389 trehalose solution of concentration higher than 500 mM, we would expect a greater change in
390 apoptosis ratio than that observed in Fig. 7. Thus, the cells in the freezing solution containing
391 trehalose would be seriously damaged after ice formation. Since this period ($t_{f2} - t_{f1}$) is longer under
392 slower cooling rate conditions, the slower cooling rates would allow more cell damage to accrue,
393 reducing the viabilities of all three cell lines. Unlike trehalose, Me_2SO can penetrate the cell and
394 reduce the osmotic pressure generated during the freezing process. Therefore, we consider that the

395 cells immersed in the freezing solution containing Me_2SO incurred little damage from additional
396 dehydration even after ice formation in the extracellular solution, resulting in their high viabilities
397 under the lower cooling rate conditions.

398 If this hypothesis is correct, the viability would be improved if trehalose were to be
399 transported into cells in the manner of a cell membrane-permeable CPA. To confirm this, we
400 compared the cooling-rate dependence of the cell viabilities in the present study with those in a
401 previous study on CHO cells expressing TRET1, a protein that passively transports trehalose (CHO-
402 TRET1 cells) [18]. Figure 9 shows that the viability of CHO-TRET1 cells frozen with 400 mM
403 trehalose was intermediate between that of CHO-vector cells frozen with trehalose and that of CHO-
404 vector cells frozen with Me_2SO below 35°C/min. This result suggests that CHO-TRET1 cells were
405 able to survive during the freezing by reducing the osmotic-pressure difference by taking up extra
406 trehalose via TRET1, thereby preventing cell damage. Since the rate of TRET1-mediated trehalose
407 uptake into cells is known to be slower than the uptake rate of Me_2SO [19][21], the viability of
408 CHO-TRET1 cells might not have been substantially improved compared to that of CHO-vector
409 cells in solution with Me_2SO.



410

411 Fig. 9. Comparison of viabilities depending on the cooling rates of CHO-TRET1 cells frozen with

412 400 mM trehalose (pre-cooling immersion for 2 h; solid circles) [18] with those of CHO-vector cells

413 with 500 mM trehalose (solid squares, CHO-vector Trehalose, corresponding to CHO-vector

414 Trehalose in Fig. 4) and with 10% Me_2SO (open squares, CHO-vector Me_2SO, corresponding to

415 CHO-vector Me_2SO in Fig. 2).

416 Another factor that may have contributed to the viability improvement in CHO-TRET1 cells

417 is the difference in the inhibitory effect of CPA on the intracellular ice crystal formation. Similar to

418 the inhibition effect of Me_2SO on the intracellular ice formation, the intracellular trehalose in CHO-

419 TRET1 cells would be expected to inhibit ice formation in the cell. However, because extracellular

420 trehalose cannot penetrate into CHO-vector cells, it cannot inhibit the intracellular formation of ice

421 crystals, and ice nuclei are formed. The slower the cooling rate, the more the ice nuclei formed inside

422 the cell grow, which would increase the damage to the cells. The difference in viabilities between

423 CHO-vector cells frozen with Me_2SO and CHO-TRET1 cells frozen with trehalose may be caused by

424 the different process of inhibition of intracellular ice formation between the two CPAs. This
425 possibility will be examined in future studies.
426
427 3.3.2. Effect of rapid cooling on the freeze–thaw viability (faster than 80°C/min)
428 At cooling rates higher than 80°C/min, both CHO-M1 and CHO-M23 cells had higher viabilities
429 than CHO-vector cells independent of the CPA used and independent of their membrane-permeable or
430 membrane-impermeable nature (Fig. 2: Me_2SO; Fig. 4: Trehalose). According to the two-factor
431 hypothesis, when the cooling rate increases, dehydration does not occur in time to prevent intracellular
432 ice crystal formation and intracellular ice crystals form, resulting in a decrease in viability. Therefore,
433 the fact that the cells overexpressing AQP showed a higher viability suggests that the dehydration
434 during the rapid cooling through AQP4 may improve the freeze–thawing viability. On the other hand,
435 it also suggests that the dehydration difference due to the osmotic-pressure difference caused by the
436 extracellular CPAs (Section 3.2.2) before ice formation had little effect on the viability under this
437 condition. This indicates that the rapid dehydration by AQP4 from the time of ice crystal formation
438 (t_{f1}) to the freezing of the solution (t_{f2}) improves viability.
439 The time required for the complete freezing of the extracellular solution at the fastest cooling
440 rate conditions was only a few seconds, as estimated by the temperature measurement (Fig. S1), so
441 the additional dehydration must have been complete within several seconds at the freezing
442 temperature, which was lower than 0°C. from Section 3.2.2, the water permeability through the

443 membrane becomes small at lower temperatures, so the lower viability of CHO-vector cells under
444 these conditions would be caused by the failure of this additional dehydration process. On the other
445 hand, water permeability through aquaporins is considered to have less temperature-dependence and
446 to be greater than that through the membrane, so a sufficient amount of additional dehydration
447 occurred during the extracellular freezing in CHO-M1 and CHO-M23 cells, resulting in the
448 improvement of their viabilities. Although this process is consistent with the two-factor hypothesis,
449 which incorporates the effect of IIF, our study reveals that the critical dehydration occurs after the
450 extracellular ice-crystal formation.

451 At this cooling rate, it is difficult to suppress IIF by intracellular CPA, so it is necessary to
452 suppress IIF by dehydration. However, since the viability was close to 0 in the cultures without added
453 CPA, it appears that both trehalose and Me_2SO functioned to protect the cell membrane and/or inhibit
454 extracellular ice crystal formation, in addition to inhibiting IIF.

455

456 3.3.3. Effect of dehydration during intermediate cooling on viability (between 35°C/min and
457 80°C/min)

458 The freeze–thaw viabilities in the intermediate cooling rate range (from 35°C/min to 80°C/min)
459 depended neither on the cell lines nor on CPAs but rather decreased with increasing cooling rate (Fig.
460 2: Me_2SO; Fig. 4: Trehalose).

461 In Section 3.3.1, we considered that the cells would be damaged by the long interval between t_{fI}

462 and t_{f2} due to increasing solute concentration. At this cooling rate, however, the time interval (t_{f1}–t_{f2}) is
463 short, at less than 2 min, so the possibility of cell damage is low. On the other hand, the brevity of
464 interval t_{f1}–t_{f2} also means less water loss during this period, so the possibility of IIF increases. If AQP4
465 is expressed, it is thought that dehydration that suppresses IIF will be possible. However, from Fig. 2
466 and Fig. 4, the difference in viability between CHO-vector and CHO-M1 or CHO-M23 at this freezing
467 rate was small compared to the measurement variation, so more data must be collected to clarify this
468 issue.
469
470 4. Conclusion
471 The two-factor hypothesis states that both intracellular ice inhibition and the dehydration from
472 cells are important for cryopreservation. However, the timing and appropriate amounts of these twin
473 contributions remain unclear. In the present study, we studied the effect of dehydration on the
474 cryopreservation using CPAs and AQP4-overexpressing cells.
475 We found that it was impossible to achieve cell viability without CPAs. Our results thus
476 clarified that a mere increase in cell-membrane permeability by AQP4 is not sufficient to improve
477 viability; the cells require the protective effects conferred by CPAs to remain viable.
478 In the presence of either membrane-permeable or membrane-impermeable CPAs, we
479 distinguished two dehydration periods during cryopreservation: before and after the extracellular ice
480 formation. Under our experimental conditions, both lines of cells in trehalose solution (impermeable

CPA) completed dehydration and were not damaged before the ice formation. This indicates that, even when the cells were dehydrated by the osmotic pressure before the ice formation, the dehydration before freezing did not severely affect their viability.

When the cooling rate was faster than 80°C/min, the AQP4-expressing cells had higher viabilities than normal cells irrespective of which CPA was used. The time period from extracellular ice-crystal formation to complete freezing of the solution was short (under 1 min) at these cooling rates, so IIF would be expected to occur in normal cells, but this could likely be avoided by dehydration using AQP. In addition, dehydration using CPAs before ice crystal formation did not affect viability. Therefore, we conclude that rapid dehydration occurring after the ice formation is an important process for cell survival.

At cooling rates below 35°C/min, the viability in the medium supplemented with a membrane-permeable CPA remained high independent of AQP4 expression, whereas that in the presence of a membrane-impermeable CPA decreased as the cooling rate decreased. These facts indicate that the intrinsic membrane water-permeability may allow sufficient dehydration for the cryopreservation during the slow freezing process. Since the apoptosis ratio was small even in the solution with CPAs during the time period before the extracellular ice formation, the cells may sustain severe damages after the extracellular ice formation.

Declaration of competing interest

There are no conflicts of interest.

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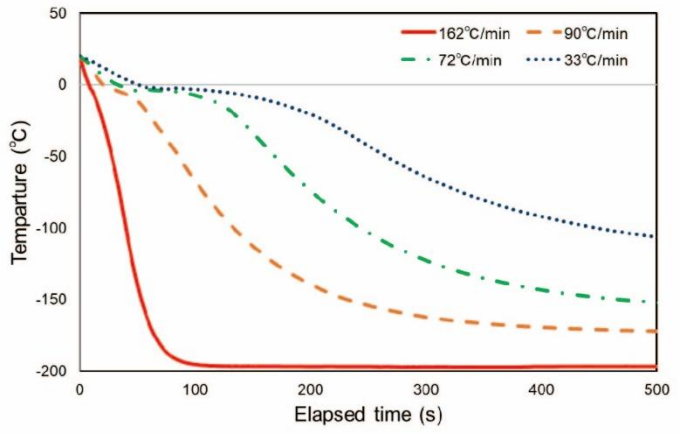
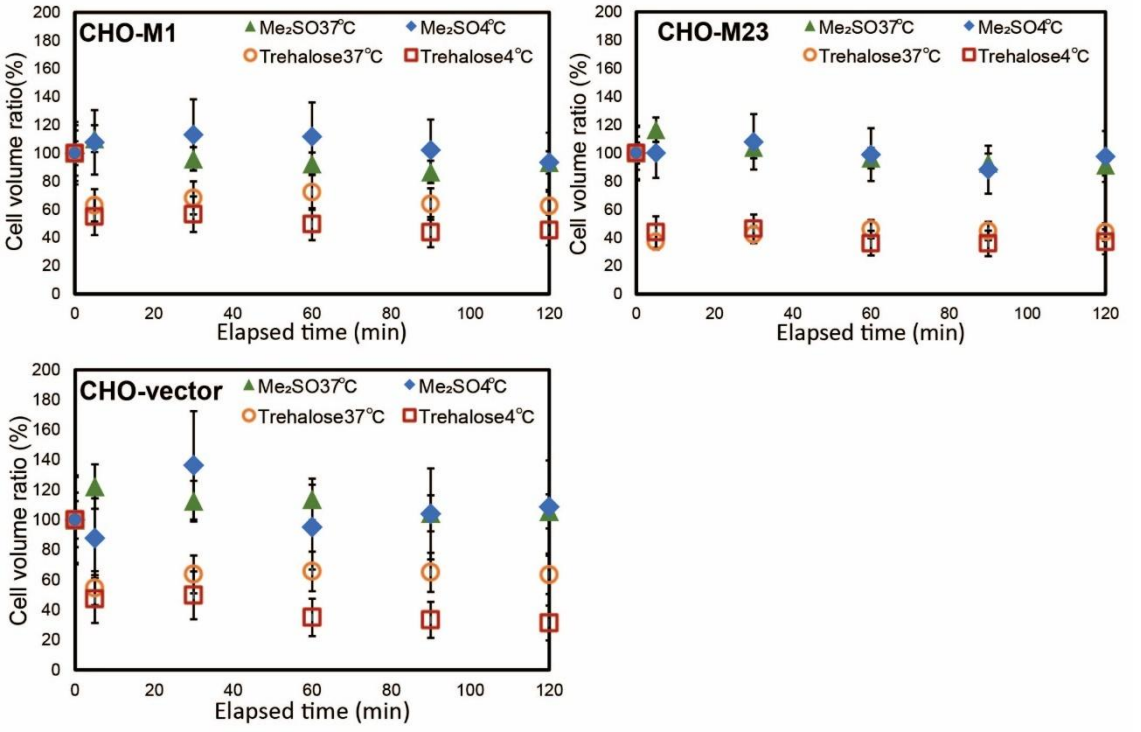
588 Supplementary materials



589

590 Fig.S1. Temperature change in 1 ml of medium containing 500 mM trehalose. The sample was frozen at 162°C/min

591 is the solid line, at 90°C/min is the dashed line, at 72°C/min is the chain line, at 33°C/min is the dotted line.



592

593 Fig.S2. Cell volume ratio for 5 minutes to 120minutes in immersion with 10% Me_2SO or 500mM trehalose at 37°C

594 or 4°C. CHO-M1 (top left), CHO-M23 (top right) and CHO-vector (bottom left)

595