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Title	Trehalose uptake and dehydration effects on the cryoprotection of CHO-K1 cells expressing TRET1
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(Appendix)

Microscopic observations of CHO-K1 cells during the immersion period

The freeze–thaw viability measurements suggested that apoptosis of the cells progressed during the pre-freezing immersion period. Moreover, more apoptosis occurred in CHO-vector cells than in CHO-TRET1 cells, and also more apoptosis occurred at longer immersion periods (Fig. 2). Thus, the apoptosis likely resulted from excess dehydration of the cells due to a high extracellular concentration of trehalose. To check this hypothesis, we used an optical microscope to observe changes to cell shapes and sizes during the immersion period. The method is as follows.

A sample in which cells are suspended in a freezing solution with a trehalose concentration of C_0 = 0, 500, and 1000 mM is placed in a culture dish, set in a CO_2 incubator, and observed using phase-contrast microscopy (IX 71) at 2-h intervals. Then the shape and size changes are observed (Fig. S1). From the images, we determine the average diameter of cells. When the shape is not circular, both the major and minor diameters are measured and the diameter of an equal volume circle is calculated by assuming an ellipsoid shape.

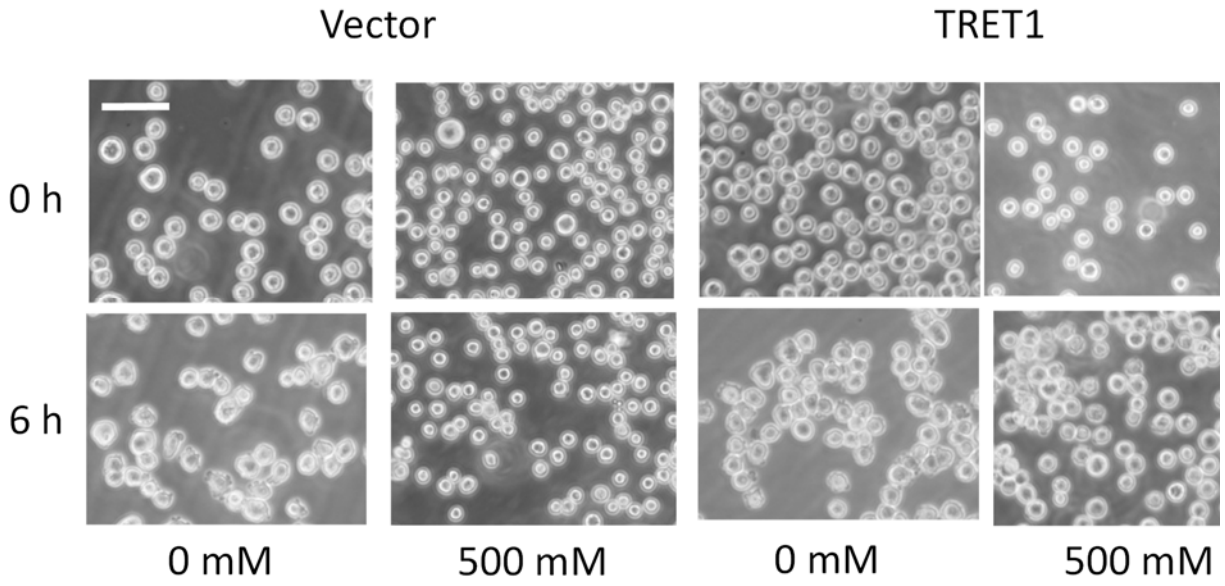


Fig. S1: Typical microscopic images of suspended cells during the immersion period. Scale bar shows 50 μm for all images.

The resulting average cell size during immersion (Fig. S2) shows that, at C_0 = 0 mM, the radii of both CHO-vector and CHO-TRET1 cells are nearly constant, or slightly increase with time. The finding suggests that the apparent size is almost constant because dehydration does not occur. At longer times, the slight apparent increase in size likely occurs due to a shape change when the cells begin to adhere to the bottom of the culture dish.

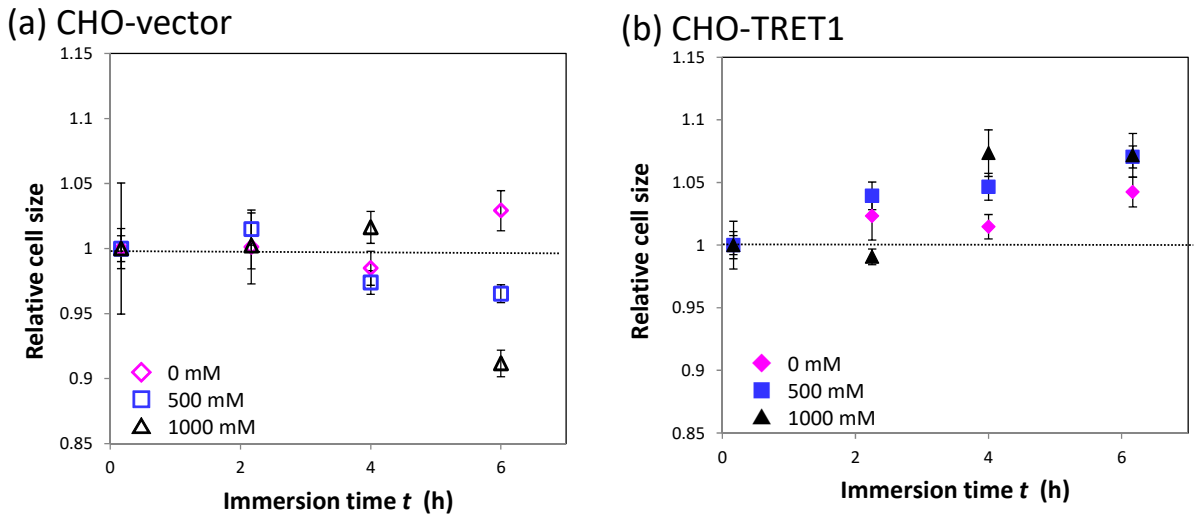


Fig. S2 : Average cell sizes of CHO-vector and CHO-TRET1 cells determined with microscopy. The extracellular trehalose concentrations are 0, 500, and 1000 mM.

The size of CHO-vector cells in the medium containing trehalose decreases with t (Fig. S2(a)), particularly with $t \geq 2$ h at higher C_0. The trend with C_0 suggests that the cell–volume shrinkage is due to dehydration. On the other hand, in CHO-TRET1 cells, the size increases at all concentrations (Fig. S2(b)). Given that the trends are similar to that observed for C_0 = 0 mM, the average size increase is likely to be mainly due to adhesion on the dish bottom. Excessive dehydration does not occur in these cases due to the small concentration difference of trehalose between inside and outside of the cells.

In addition, the dehydration rate of CHO-vector cells $\delta V/V_0$, estimated using C_0 and Eq. (5) in section 4.1, is linearly correlated with the viability after 6-h immersion, defined as $\eta^A{}_{V(6h)} = 1 - A_{V(6h)}$, with $R^2 = 0.842$ (Fig. S3). The correlation suggests that during the immersion period, CHO-vector cells are dehydrated due to the osmotic pressure of extracellular trehalose, whereby the apoptosis of the cell progresses. However, the actual size of the cells (Fig. S2(a)) does not shrink nearly as much as that from Eq. (5) (or shown in Fig. S3). Possible reasons for this large discrepancy include 1) the cell had not reached isotropic equilibrium at the observation time, 2) the cell skeleton keeps its external shape unchanged while the intracellular water exits, 3) the cell's shape flattens as it shrinks, and 4) some cell–volume loss is due not only to dehydration, but also to apoptosis, which is caused by actomyosin contraction [51].

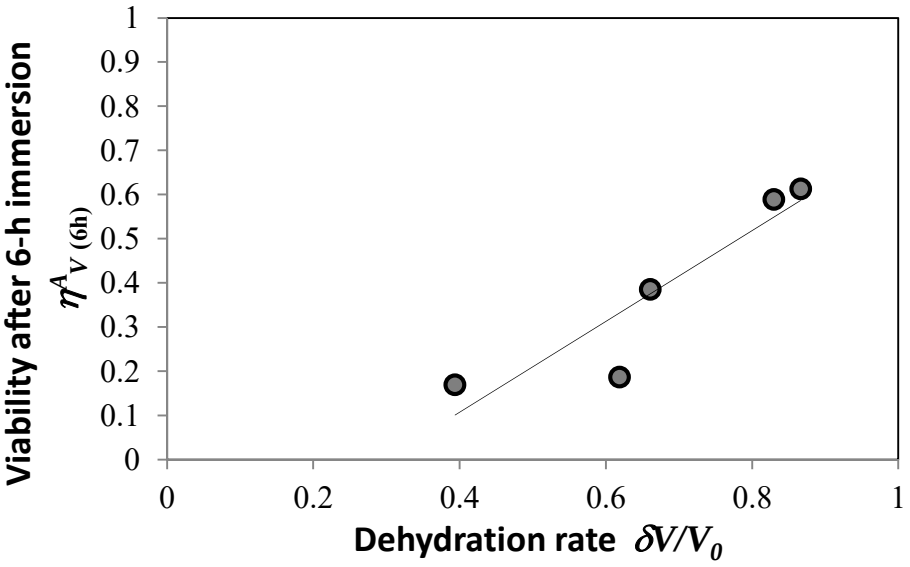


Fig. S3: Relation between viability of CHO-vector cells during 6-h immersion $\eta^A{}_{V(6h)}$ and dehydration ratio $\delta V/V_0$. $\eta^A{}_{V(6h)}$ is estimated from the apoptotic ratio A_V at $t = 6$ h, and $\delta V/V_0$ is estimated from the relation of C_0 and Eq. (5).